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Investigation of trypanothione synthetase of *Leishmania infantum* as a potential target for new anti-parasitic drugs

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Abstract

Leishmania infantum is a protozoan parasite of the Trypanosomatidae family, responsible for human and canine leishmaniasis in Mediterranean countries. Control of these vector-borne diseases is unsatisfactory and new chemotherapeutics are urgently needed. Trypanothione biosynthesis, owing to its unique and essential character, is regarded as an attractive target for therapeutic intervention. Trypanothione is a bis-(glutathionyl)spermidine conjugate, responsible for redox homeostasis in trypanosomatids. It is synthesized by the sequential addition of two molecules of glutathione to a spermidine molecule. Trypanothione synthetase (TRYS), which catalyzes both conjugation steps, has no counterpart in mammals and is essential to *Trypanosoma brucei*. This scenario is somewhat different in *L. infantum*, which harbors one additional enzyme mono(glutathionyl)spermidine synthetase or GSPS capable of driving the first step of trypanothione biosynthesis. Since mono(glutathionyl)spermidine can replace some metabolic functions of trypanothione *in vitro*, the actual significance of *TRYS* is still disputed in *GSPS*-harboring trypanosomatids. This work aimed at clarifying this issue by functionally characterizing both *TRYS* and *GSPS* in *L. infantum* promastigotes (insect stage) and amastigotes (mammalian stage), employing a classical gene replacement strategy. Concerning *TRYS*, elimination of both alleles in promastigotes was only possible upon complementation with an extrachromosomal copy of the gene. Maintenance of this episome for 6 months in the absence of drug pressure proved that *TRYS* is crucial and cannot be replaced by *GSPS*. Work is on going to assess *TRYS* essentiality in amastigotes. In parallel, we have initiated the chemical validation of TRYS using a N^5-substituted paullone (FS-554) that irreversibly inhibits the enzyme *in vitro*. We observed that the leishmanicidal effect of FS-554 towards promastigotes and intramacrophagic amastigotes correlated with TRYS expression levels, confirming that this enzyme can be targeted by drug-like compounds in the cell context. In what regards *GSPS*, production of homozygous knockouts is still underway to be used in future work.

Key words: *Leishmania infantum*, Trypanosomatidae, Trypanothione, Trypanothione synthetase, Target validation.

Resumo

Leishmania infantum é um parasita protozoário, da família Trypanosomatidae, causador da leishmaniose humana e canina em países Mediterrânicos. As terapias usadas para esta doença são ineficazes, sendo urgente descobrir novas fórmulas leishmanicidas. Devido ao seu carácter único e essencial, a via biossintética de tripanotiona, é considerada um bom alvo terapêutico. Tripanotiona é um ditiol, responsável pelo equilíbrio redox em triponassomatídeos, sintetizado pela adição sequencial de duas moléculas de glutationa a uma de espermidina. A enzima tripanotiona sintetase (TRYS), que catalisa estes dois passos de conjugação, não existe em mamíferos e é essencial em *Trypanosoma brucei*. Em *L. infantum*, no entanto, existe outra enzima, a mono(glutationil)espermidina (GSPS) capaz de catalisar o primeiro passo de síntese. Dado que a mono(glutationil)espermidina desempenha algumas das funções metabólicas da tripanotiona, a relevância da TRYS em tripanossomatídeos que expressam GSPS é discutível. Neste trabalho procurámos clarificar esta questão, tendo para isso procedido à caracterização funcional da TRYS e da GSPS em *L. infantum*, por substituição homóloga dos respectivos genes. No que respeita ao gene *TRYS*, verificámos que a eliminação dos dois alelos só foi possível após complementação dos parasitas com uma cópia episómica do mesmo. Este episoma conservou-se nos parasitas, mesmo após 6 meses de cultura na ausência de qualquer pressão para o manter, que não fosse a necessidade de expressar TRYS. Este resultado demonstrou que a TRYS é essencial para o estadio de insecto de *L. infantum*, sem que a GSPS compense a sua falta. A mesma estratégia servirá para avaliar o carácter crucial da TRYS no estadio de mamífero. Paralelamente, iniciámos a validação química da TRYS usando um composto da família química das paulonas (FS-554), que inibe a enzima irreversivelmente *in vitro*. Observámos que uma maior expressão da TRYS confere mais resistência à FS-554, confirmando que é possível interferir quimicamente com esta enzima no contexto biológico. Relativamente à GSPS, estamos a produzir parasitas "knockouts", não podendo concluir sobre a sua relevância funcional.

Termos chave: *Leishmania infantum*, Trypanosomatidae, Tripanotiona, Tripanotiona sintetase, Alvo terapêutico.

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Abbreviations

AdoMet	S-adenosylmethionine
AdoMetDC	S-adenosylmethionine decarboxylase
ADP	Adenosine diphosphate
AMP	Beta-lactamase
ARG	Arginase
AscPX	Ascrobate peroxidase
ATP	Adenosine triphosphate
AIDS	Acquired Immune Deficiency Syndrome
bp	Base pairs
BSA	Bovine Serum Albumin
BSD	Blasticidin S deaminase
Cf	*Crithidia fasciculata*
CHAP	Cysteine, histidine-dependent amidohydrolase/peptidases
CL	Cutaneous Leishmaniasis
CO_2	Carbon dioxide
cTXNPx	Cytosolic tryparedoxin peroxidase
dAdoMet	Decarboxylated AdoMet
DAPI	4',6-diamidino-2-phenylindole
DCL	Diffuse Cutaneous Leishmaniasis
dCTP	Deoxycytidine triphosphate
DFMO	Eflornithine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Ec	*Escherichia coli*
EC_{50}	Half maximal effective concentration
FBSi	Inactivated fetal bovine serum
g	gram
GCL	Glutamate cysteine ligase
gRNA	Guidance RNA
GPx	Glutathione peroxidase-like enzyme
GR	Glutathione reductase
GSH	Glutathione
GSP	Mono(glutathionyl)spermidine
GSPS	Glutathionyl spermidine synthetase
gsps$^{+/-}$	*L. infantum Δgsps::BSD/GSPS* mutants
G418	Geneticin
h	Hours
Hepes	4-(2hydroxyethyl)-1-piperazineethanesulfonic acid
HYG	Hygromycin phosphotransferase
IBMC	Institute for Molecular and Cell Biology
IC_{50}	Half maximal inhibitory concentration
IFAT	Indirect Fluorescence Antibody Test
IgG	Immunoglobulin G
kbp	kilo base pairs

kDa	kilo Dalton
kDNA	Kinetoplast DNA
LB	Lysogeny Broth
Li	*Leishmania infantum*
LC_{50}	Median lethal dose
Lm	*Leishmania major*
M	Molar
MCL	Mucocutaneous Leishmaniasis
min	Minutes
ml	mililiter
mM	milimolar
mRNA	Messenger RNA
mTXNPx	Mitichondrial tryparedoxin peroxidase
mV	milivolt
NCS	Non-coding sequence
NMRI	National Marine Research Institute
NO_2^-	Nitrite
nM	Nanomolar
ODC	Ornithine decarboxilase
O/N	Over Night
ORF	Open Reading Frame
PAC	Puromycin N-acetyl-tranferase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHLEO	Phleomycin hydrolase
RiboR	Ribonucleotide reductase
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RPMI	Royal Park Memorial Institute
Sec	Seconds
SL	Spliced Leader
SOC	Super Optimal broth with Catabolite repression
SPDS	Spermidine synthetase
Tc	*Trypanosoma cruzi*
Tb	*Trypanosoma brucei*
TR	Trypanothione reductase
tRNA	Transfer RNA
TRYS	Trypanothione synthetase
$trys^{+/-}$	*L. infantum* Δ*trys*::*HYG/TRYS* mutants
$trys^{-/-}$/+*TRYS*	*L. infantum* Δ*trys*::*HYG*/Δ*trys*::*PHLEO* [pTEX-*NEO*-*TRYS*] mutants
$T(SH)_2$	Trypanothione
TXN	Tryparedoxin
U	Unit
UMSBP	Universal minicircle sequence binding protein
v	Volume
VL	Visceral Leishmaniasis
w	Weight
WHO	World Health Organization
WT	Wildtype

Chapter 1

Introduction

1. Leishmaniasis

1.1. The disease

Leishmaniasis is a set of vector-borne diseases with different clinical manifestations, caused by several species of the protozoan *Leishmania* genus. Although frequently regarded as a disease of dogs in developed countries, it also affects other mammals, including man, causing victims primarily in the tropical and sub-tropical areas of the world. Human leishmaniasis is responsible for more than 50,000 fatalities per year and about 350 million people in 88 countries all around the world are at risk of contracting the disease. At present, over 12 million individuals are estimated to be infected, although the precise number remains elusive due to inadequate diagnostic and lack of disease reports. Despite these numbers, the fact that majority of cases occur mostly in poor areas of the world renders this disease forgotten by the developed countries [*1*].

Leishmaniasis can be transmitted from human to human (anthroponotic transmission) or from other mammals to humans (zoonotic transmission). The vector responsible for transmission is the female sandfly of the *Phlebotomus* (Old world) or *Lutzomyia* (New world) genera. Approximately 30 different species transmit leishmaniasis and a single bite is enough to establish infection. Depending on the *Leishmania* species that are transmitted to man, the disease can have different clinical manifestations, either as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) or visceral leishmaniasis (VL) [*2*].

Cutaneous leishmaniasis is the most common form of the disease in humans and, despite not being lethal, it dramatically reduces the life quality of patients. This manifestation of the disease is characterized by skin lesions that can be localized (nodules or ulcers) or non-localized (diffuse cutaneous leishmaniasis or DCL). In immunocompetent individuals, infection can be controlled and skin lesions usually heal spontaneously leaving trifling scars [*3*].

Mucocutaneous leishmaniasis affects mainly mucosal areas, inducing destructive inflammation of nasal, oral and throat mucosal membranes. Unlike CL, this manifestation of the disease is not self-healing and sometimes, even upon a successful treatment, it leaves permanent disfiguring scars, thus exposing infected individuals to social discrimination and shame [*4*].

Visceral leishmaniasis is by far the most aggressive form of the disease and may even be fatal if not properly treated. It consists on a systemic infection that can virtually affect any macrophage-containing organ, even though liver, spleen, bone marrow and lymph nodes are preferentially infected. Common symptoms include high fever, weight loss, anemia, abdominal pain, diarrhea and swelling of the liver and spleen [*5*]. Establishment of a VL infection does not always translate into clinical manifestations. Individuals with a fragile immune system, such as children and immunosuppressed individuals (*e.g.* AIDS patients) are usually more susceptible to display symptoms. The latter have, actually, been pointed out by the World Health Organization

(WHO) as a major problem in VL control. Indeed, while many *Leishmania*-infected individuals remain asymptomatic, HIV-infected patients are 100 to 2300 times more susceptible to develop active VL. Likewise, VL accelerates HIV replication and progression [*6*]. Apart from being severely affected, HIV-*Leishmania* infected patients also contribute to VL dissemination to non-endemic areas. This scenario is of particular concern in countries of the Mediterranean basin, such as Portugal [*7*].

The incidence of the different manifestations of the disease around the World is tightly correlated with the geographical distribution of the different *Leishmania* species (Table 1.1). Leishmaniasis affects mainly poor countries and is frequently associated with poverty, malnutrition, illiteracy, weakness of the immune system and lack of resources, having a pronounced socio-economic impact in already debilitated communities. The intense human migration, added to alterations of the geographical distribution of the insect vector, propelled by climate changes, may lead to disease dissemination to non-endemic areas. Still, in developed countries the cases of human leishmaniasis remain rare so far [*1,8*]. In the developed areas of the world leishmaniasis has higher impact in veterinary health. This is the case of Portugal, as well as other European countries of the Mediterranean basin, where there are numerous cases of canine leishmaniasis caused by *Leishmania infantum*. Importantly, by acting as main reservoirs, dogs increase the risk of human transmission due to their proximity to the population [*9*].

Table 1.1. Geographical distribution of human leishmaniasis

	Species	Disease manifestation	Geographical distribution
Old World	*L. infantum*	VL	Mediterranean regions, Northern Africa, central Asia and northwest China
	L. donovani	VL	China, Northern and East Africa
	L. major	CL	Northern Africa, Middle East, East Africa and India
	L. tropica	CL	Mediterranean regions, Afghanistan, northern Africa, Middle East and India
	L. aethiopica	CL	Ethiopia, Kenya and Uganda
New World	*L. chagasi*	VL	Central and South America
	L. mexicana	CL	Central and South America and south regions of USA
	L. amazonensis	CL	South America
	L. braziliensis	MCL	Central and South America

VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis. Adapted from Romao [*10*]**.**

1.2. Disease control

At present all efforts to control leishmaniasis have failed the level of demand and human beings are currently lagging behind in the battle against this disease. To date there are no human vaccines to prevent the disease and the available chemotherapeutic drugs are unsatisfactory [reviewed in *11*,*12*]. Means to control leishmaniasis in endemic areas include the activity of humanitarian agencies, which promote educational and interventional programs. Examples of some of the measures applied in affected nations are control of vector population, elimination of reservoir animals and campaigns to raise the population awareness with respect to disease symptoms [*1*]. Additionally, the development of new diagnosis techniques has also been encouraged as an important tool to control the disease, as the currently used practices involve invasive and risky procedures that are problematic to employ in a third world environment [*13*].

In what concerns chemotherapeutic treatment of leishmaniasis, it currently relies on highly toxic antimonial compounds that have been used as first-line drugs since 1945. The most commonly used are the trivalent antimony complexes Stibophen®, Repodral®, Fuadina® and the pentavalent antimony complexes Pentostan®, Glucantime® and Solustibosan®. Furthermore, non-antimonial compounds are also used in leishmaniasis treatment as second-choice drugs. The most used are Lomidina® (pentamidine), amphotericin B (a macrolide antibiotic) and miltefosine (a phospholipid derivative) [*3,14*]. A lipid formulation of amphotericin B has been pointed out as one of the best options in VL treatment. Unfortunately, its prohibitive price and parenteral route of administration rule out the application of this drug in poor developing countries [*15,16*].

Despite the collection of leishmanicidal drugs available, none of them is fully satisfactory. In fact they all suffer from either poor efficiency, high toxicity, long-term treatment or inconvenient mode of administration. Moreover, in many cases the emergence of drug-resistant parasites also renders some of the currently used drugs inadequate for leishmaniasis treatment [*17*]. Development of a human vaccine arises as an appealing alternative to the chemotherapeutic approach. However, all attempts to create an efficient and safe vaccine have proved unsuccessful so far. The first prophylactic measure against leishmaniasis, known as "Leishmanization", was developed around 1940 and had been used for over 60 years in many countries. It consisted in inoculation of virulent *Leishmania* parasites from a cutaneous lesion into healthy individuals. Although still practiced in Uzbekistan, this procedure proved to have major adverse effects and has long been abandoned in majority of countries. After "Leishmanization", first-generation vaccines were initially designed to produce a marked immune response in the host by injecting live attenuated parasites (*i.e.* parasites that can infect but are not pathogenic) or killed parasites. Second- and third-generation vaccines are more sophisticated formulations consisting of purified fractions from *Leishmania* and purified DNA, respectively. Some of these vaccines had their important milestones, but none have successfully fulfilled the requirements necessary for a tradable human vaccine. Advances made in canine leishmaniasis treatment led to favorable expectations regarding the development of a human vaccine [reviewed in *18,19*]. To date there are already two registered vaccines to prevent canine leishmaniasis, namely Leishmune® in Brazil and Canileish® in Europe [*20,21*].

2. *Leishmania*

2.1. Taxonomy

Leishmania spp. belong to the order Kinetoplastida, a group of the protozoan phylum that includes a number of obligatory parasites affecting a variety of living organisms, including plants, insects and vertebrates [*22*]. Some of these parasites, in particular those of the

Trypanosomatidae family are responsible for potentially lethal vector-borne diseases with importance in human and veterinary health. The *Leishmania* genus, along with the *Trypanosoma* genus, represent the two major human pathogens of this family. Currently, more than twenty *Leishmania* species have been described and divided into two subgenera: *Leishmania Leishmania*, which contains both New world and Old world species, and *Leishmania Viannia*, including only New world species. As for the *Trypanosoma* genus, it also includes two pathogen species that cause potentially lethal diseases, namely *Trypanosoma cruzi*, the causative agent of American trypanosomiasis (also known as Chagas' disease), and *Trypanosoma brucei*, responsible for human African trypanosomiasis (commonly known as sleeping sickness) and for the Nagana disease in cattle [*23*,*24*].

2.2. Biology

Kinetoplastids present features common to other eukaryotic cells, like plasma membrane, nucleus, nucleolus and ubiquitous organelles. However, these organisms display peculiar traits that distinguish them from higher eukaryotes, as detailed next.

Kinetoplastids are unique for harboring a DNA agglomerate within their single mitochondrion, known as kinetoplast or kDNA. This disk-like structure consists of a network of two types of circular DNA, a few dozen maxicircles (20-40 kbp) and several thousands minicircles (0,5-10 kbp). Maxicircles are responsible for encoding ubiquitous subunits of the respiratory chain, as well as two rRNAs molecules, whereas minicircles encode guidance RNAs (gRNA) that are implicated in the editing of maxicircles' transcripts, involving insertion or deletion of uridine bases [*25*].

The mitochondrion of kinetoplastids is also unusual for existing as a single, elongated ultra-structure that extends along the longitudinal axis of the parasite body. Although it comprises the same function of mitochondria in higher eukaryotes (*i.e.* energy production through oxidative phosphorylation), interactions between enzymes of the electron-transfer chain differ [*26*]. Moreover, at least in *T. brucei*, there are two terminal oxidases involved in the electron respiratory chain, the classical cytochrome oxidase and an alternative cytochrome-independent oxidase [*27*]. Like in other eukaryotes, most of the proteins of the mitochondrion are synthesized in the cytosol and are then imported into this organelle. However, the few proteins that are synthesized inside the mitochondrion require recruitment of tRNAs, which are not encoded by the mitochondrial genome of kinetoplastids [*28*].

Another distinguishing feature of kinetoplastids is the presence of peroxisome-like organelles, known as glycosomes. Except for catalase, these organelles comprise most of the enzymes present in peroxisomes of higher eukaryotes, namely those involved in β-oxidation of fatty acids, biosynthesis of pyrimidines and purine salvage pathways. What renders glycosomes

unique is the compartmentalization of part of the glycolytic pathway (glucose to glycerate 3-phosphate conversion), which in higher eukaryotes occurs in the cytosol. Compartmentalization of glycosis in these organelles may allow a more efficient and independent control over glucose consumption in situations of unfavorable ATP/ADP ratio inside cells [*29*].

In what concerns molecular biology, trypanosomatids possess a highly plastic genome, characterized by fluctuations in the number and size of chromosomes, as well as by the amplification of large regions of the genome, a phenomenon that is usually associated with drug resistance [*30*,*31*]. In addition, during mitosis nuclear membrane of trypanosomatids is not disrupted and chromatin condensation is not observed during chromosome segregation [*32*]. Also notably, DNA transcription in these organisms presents some exclusive traits with miscellaneous characteristics between eukaryotes and prokaryotes. In particular, genes without any functional relation are transcribed as large polycistronic units that undergo a *trans*-splicing mechanism before transduction. In this process a small capped RNA (spliced leader or SL) is added at the 5' end of each coding region, followed by a non-specific sequence polyadenylation at the 3'-end. Intriguingly, trypanosomatids lack classical eukaryote or prokaryote promoter regions and to date no consensus regions have been found that can be regarded as promoters. This fact, along with the polycistronic nature of their mRNA, deprives trypanosomatids from transcription control of individual genes. Usually, transcription initiates between two gene clusters with divergent transcription directions, *i.e.* towards or away from the telomeres. Even though there are no obvious promoter sequences in the genome of trypanosomatids, untranslated regions flanking coding sequences are known to be important to regulate gene expression [reviewed in *33*,*34*].

Finally, the thiol metabolism in these organisms presents many differences towards other eukaryotes. Briefly, they possess a unique conjugate of glutathione (GSH) and spermidine, known as trypanothione that takes over most of the functions of GSH in higher eukaryotes. The unique characteristics of this thiol and how these can be explored for drug development will be detailed in section *3.2.* of the Introduction.

2.3. Life cycle

Leishmania, as well as other members of the Trypanosomatidae family, have a digenetic life cycle during which they suffer major morphological and biochemical modifications (Figure 1.1) that are associated to two distinct life stages: *i)* the promastigote, an elongated, extracellular single flagellated form that resides inside the alimentary tract of the vector, and *ii)* the amastigote, a non-motile aflagellated round shaped form that thrives inside macrophages of the host.

When an insect vector feeds on the blood of an infected host it ingests amastigote-containing macrophages and initiates the infection cycle. Inside the insect digestive tract, amastigotes are released from macrophages and immediately begin to differentiate into promastigostes. On the subsequent 6-10 days, significant morphological and biochemical changes occur, ultimately culminating in the migration of the promastigote infective form (metacyclic promastigotes) into the thoracic midgut and proboscis of the sandfly. By this time, if the infected vector feeds on a mammalian host, it will deposit several thousands of metacyclic promastigotes into its skin [*35*]. Parasite inoculation immediately triggers an immune response with the concomitant recruitment of phagocytic cells, like neutrophils, dendritic cells and macrophages, to the site of inoculation. *Leishmania* can subsequently be phagocytized by any of these cells, through a receptor-mediated phagocytosis mechanism. The role of neutrophils and dendritic cells during *Leishmania* invasion is still disputed. However, it is within mononuclear phagocytes that the parasite replicates and persists [*36*,*37*]. Inside these cells, parasites reside in phagolysosomes (the outcome of the fusion of a phagosome with a lysosome), where they revert into amastigotes and multiply by binary fission. Massive multiplication in these acidic vacuoles eventually leads to macrophage burst, after which parasites can be phagocytized again by other macrophages, in this way perpetuating the amastigote cycle inside the host [*38*].

For research purposes, both *Leishmania* life cycle stages can be cultured *in vitro*, in appropriate medium and conditions. Promastigotes are cultured at 25 ºC in a neutral buffered medium (pH=7), reflecting the environment found in the alimentary tract of the insect vector, whereas amastigotes can be grown axenically in acidic medium (pH=5) at 37 ºC, mimicking the conditions inside phagolysosomes. Alternatively, the amastigote stage can also be studied intracellularly in *ex vivo* infections of macrophage cultures.

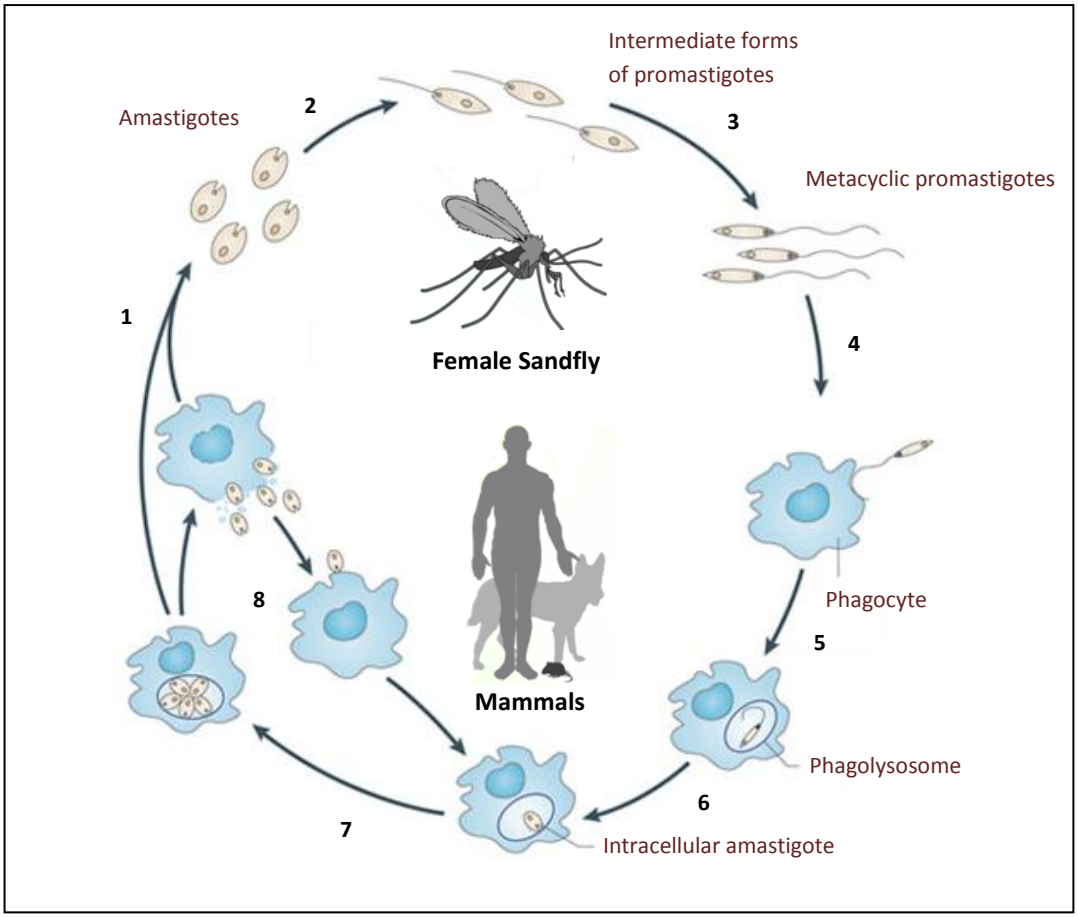

Figure 1.1. The *Leishmania* life cycle. During a blood meal, the female sandfly ingests macrophages containing amastigotes that are released in the insect midgut (1). Amastigotes differentiate into promastigotes (2) and these go through a series of differentiation phases that culminate in metacyclic infective forms (3). The infected sandfly feeds on a mammalian host, injecting metacyclic promastigotes into the host skin (4), where they are internalized by phagocytic cells (5). Parasite-containing phagosomes fuse with lysosomes, forming phagolysosomes, wherein promastigotes revert into amastigotes (6) and begin to replicate by binary fission (7), leading to the burst of phagocytes. Released amastigotes can be taken up by other phagocytes, in this way perpetuating the amastigote stage inside the mammalian host (8). Adapted from Kaye *et al.* [*39*].

3. Quest for new chemotherapies against leishmaniasis

3.1. Strategies for the discovery of new drugs

The unsatisfactory traits of current therapies against leishmaniasis, together with disease dissemination and the increasing reports of drug-resistant parasites, renders the development of new leishmanicidals a mandatory issue. The urgency to find new drugs arises as a major concern to researchers all around the world and efforts to increase the drug pipeline has been made in the last few years, mostly at the academic level. Two main strategies have been applied to pursuit this goal. One employs high-throughput drug screening trials, sometimes using compounds already established in the market, in order to find antiparasitic drugs. Selected compounds can then be exploited to understand their specific mode of action and, if necessary

and possible, considered to be chemically modified to increase target specificity [*40*]. The other strategy, known as rational drug design, consists in identifying molecular targets that are essential to parasite survival, so that their depletion or inactivation leads to parasite death. In this context, a deep knowledge of the parasite biochemistry is crucial to find a good candidate that can be inhibited by drug-like compounds inside the macrophages of the mammalian host. This approach employs several interdisciplinary techniques, from molecular/cell biology to biochemistry and computer sciences, to assess the likelihood of a molecule to be used as drug target. The appraisement of a certain gene candidate to serve as a drug target includes its validation both at the genetic and the chemical level, as detailed next.

Genetic validation consists in evaluating the essentiality of a given gene during the life cycle of the parasite, resorting to common molecular and genetic techniques. Abrogation of gene expression is pursued and associated phenotypic changes evaluated. In the case of most *Leishmania spp.*, where interference RNA or inducible expression systems are either absent or still deficient, this is usually achieved by a classical gene knockout strategy. This consists in replacing the gene of interest by other that confers resistance to a toxic drug. Since *Leishmania* are diploid organisms, elimination of both alleles requires two rounds of homologous recombination with different drug resistance markers. Following this approach, there are two possible outcomes: *i)* parasites grow normally throughout their complete life cycle, with no apparent phenotypic alterations, indicating that the target gene is not essential for parasite survival, hence not suitable as drug target; *ii)* elimination of both gene alleles is not compatible with parasite life, indicating that the gene may be further exploited for therapeutic purposes [*41*].

Chemical validation is a complement of the genetic validation. It consists of using a known inhibitor of the target molecule to verify whether its inactivation affects parasite viability. This approach addresses important "druggability" issues, namely the possibility of blocking the target molecule with low effective concentrations (EC_{50}) of drug like-compounds in the cell context to impair parasite survival.

In short, following a rational drug design strategy, a gene to be regarded as a valid target for drugs has to fulfill the requirements of essentiality, exclusiveness and susceptibility to inhibition in the medically relevant life stage of the parasite.

3.2. The trypanothione system as a potential target for drugs

In the quest to find new drug targets, the thiol system of trypanosomatids has received much attention in the last two decades owing to its unique character. Unlike most living organisms, trypanosomatids lack the conventional GSH/GSH-reductase (GR) redox couple that is responsible for maintaining a reducing intracellular environment. Instead, this function is

largely accomplished by the trypanosomatids' unique thiol, N^1,N^8-bis(glutathionyl)spermidine, also known as trypanothione [$T(SH)_2$]. This molecule is the major low molecular mass thiol in trypanosomatids and plays a pivotal role in many essential metabolic pathways [reviewed in *42*].

Trypanothione was first described in 1985 by Alan Fairlamb and co-workers in *C. fasciculata*, a member of the trypanosomatid family that is noninfectious to mammals [*43*]. It is a small dithiol, formed by one spermidine molecule (a cationic polyamine) with two GSH molecules covalently bound to its terminal amino groups. Originally considered as a co-substrate of GR, the actual role of $T(SH)_2$ was only elucidated upon the finding that typanosomatids do not possesses GR and that, instead, harbor a trypanothione reductase (TR) enzyme. It is now well established that in trypanosomatids the $T(SH)_2$/TR system replaces most of the functions played by the GSH/GR redox couple in mammalian cells. Even though GSH is also present in trypanosomatids, it is thought to play a minor role in the redox metabolism of parasites, as all enzymes that in mammals depend on GSH are either absent in these organisms (*e.g.* GR and selenium-containing GSH peroxidases) or preferentially utilize $T(SH)_2$ as reducing substrate [*44*,*45*].

When compared with GSH, $T(SH)_2$ is a more reactive, positively charged molecule capable of spontaneously reducing different biological disulfides [*46*]. This higher reactivity is not attributed to its redox potential (-242 mV), which does not significantly differ from that of GSH (-230 to -250 mV) [*47*]. Rather, it is the pK_a value of the thiol groups of trypanothione (7.4) that contributes to its increased reactivity relative to that of GSH (pK_a of 8.7-9.2) [*48*]. This is explained by the fact that thiol-disulfide exchange reactions are highly favored when the pK_a value of the thiol is proximal to the pH value of the surrounding medium. Moreover, the dithiol nature of $T(SH)_2$ allows the formation of an intramolecular disulfide bond that is kinetically favored relative to intermolecular disulfide bonds, as occurs in oxidized GSH (GSSG) [*49*].

Trypanothione is directly or indirectly involved in many metabolic processes (Figure 1.2), from redox state regulation (by maintaining thiol redox homeostasis) to elimination of toxic compounds (including detoxification of reactive oxygen species and extrusion of toxic compounds via a trypanothione-S-transferase) [*50-52*] and DNA replication [by providing reducing equivalents to ribonucleotide reductase and to the universal minicircle sequence binding protein (UMSBP)] [*53*,*54*]. Many of these $T(SH)_2$-dependent pathways have been genetically validated as drug targets in the past [*42*]. Accordingly, inhibition of trypanothione biosynthesis should disturb many of these vital metabolic routes, eventually leading to parasite death. This, added to the unique character of this thiol, renders the $T(SH)_2$ biosynthesis pathway an attractive option when considering potential drug targets for trypanosomatids.

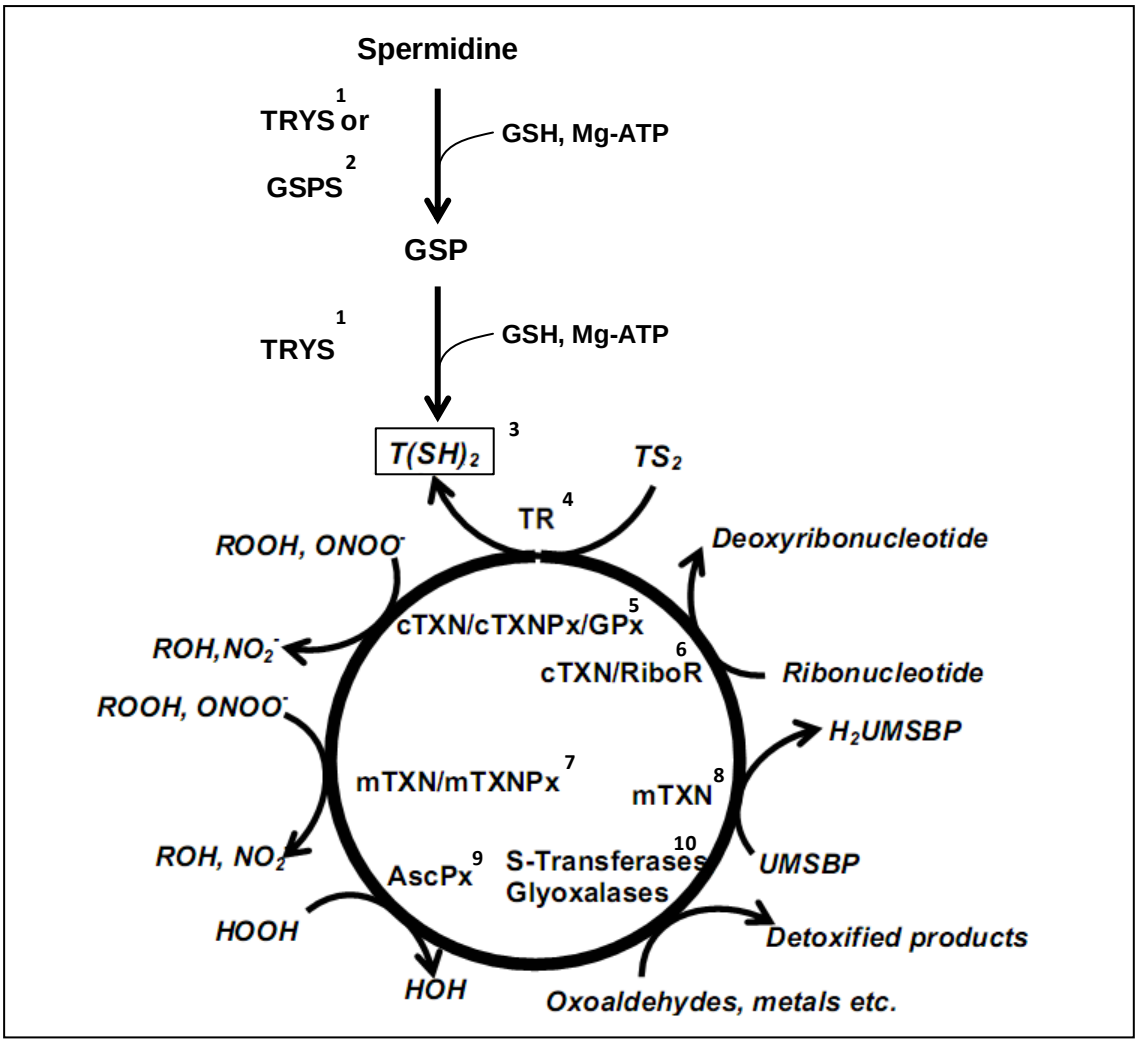

Figure 1.2. The trypanothione system.
1. *De novo* synthesis of trypanothione [$T(SH_2)$] is achieved by the ATP-dependent conjugation of two GSH molecules to spermidine. This reaction is catalyzed by trypanothione synthetase (TRYS), which in *T. brucei* was proven to be essential for parasite survival [*55*,*56*].
2. The first GSH conjugation can also be catalyzed by the GSPS enzyme to produce N^1 or N^8-mono(glutathionyl)spermidine (GSP). This compound have been suggested to replace some of the $T(SH)_2$ functions [*57-59*].
3. Trypanothione is the major thiol of trypanosomatids and is essential to many vital pathways. It delivers reducing equivalents to the enzymes of this system.
4. Trypanothione reductase (TR) is a flavin-containing disulfide reductase responsible for maintaining $T(SH)_2$ in the reduced state. This enzyme was shown to be essential for the survival of several trypanosomatids and is one of the most exploited drug targets in these organisms [*60*].
5. Trypanothione reduces cytosolic tryparedoxins (cTXN) [*61*], which provide the reducing equivalents to the peroxiredoxin-type tryparedoxin peroxidases (cTXNPx) [*62*,*63*] and to non-selenium glutathione peroxidase-like enzyme (cGPx) [*64*], the enzymes responsible for detoxification of a broad spectrum of peroxides. cTXN was shown to be essential to trypanosomatids survival [*65-67*].
6. Trypanothione directly, or via cTXN, reduces ribonucleotide reductase (RiboR), which is involved in DNA synthesis and repair, therefore implicating $T(SH)_2$ in cell division [*53*].
7. A mitochondrial tryparedoxin (mTXN) provides reducing equivalents to the mitochondrial TXNPx (mTXNPX) and GPx (mGPx), which is responsible for elimination of reactive oxygen species [*50*].
8. Trypanothione, via mTXN, reduces the universal minicircle sequence binding protein (UMSBP), which is indispensable for the replication of kDNA [*54*].
9. Trypanothione reduces ascorbate, which serves as substrate of ascrobate peroxidase (AscPX) [*68*,*69*].
10. Trypanothione is involved in detoxification of xenobiotics via the activity of an S-transferase [*51*,*52*], oxoaldehydes via the glyoxalase system and also in detoxification of heavy metals [*70*,*71*].
This scheme was adapted from Flohé 2009 [*42*].

3.3. Trypanothione biosynthesis

As mentioned above, $T(SH_2)$ is formed by one spermidine and two GSH molecules. The metabolic pathways of these two components are similar to those of mammalian cells. In the case of GSH, this small thiol is synthesized by the sequential conjugation of cysteine, glutamic acid and glycine residues, involving two distinct enzymes, namely glutamate cysteine ligase (GCL) and glutathione synthetase. As for spermidine, its biosynthesis starts with L-arginine conversion to L-ornithine by arginase (ARG), followed by decarboxylation by ornithine decarboxilase (ODC) to yield putresine. In parallel, S-adenosylmethionine (AdoMet) is also decarboxylated by the S-adenosylmethionine decarboxylase (AdoMetDC) to originate decarboxylated AdoMet (dAdoMet). Finally, putresine and dAdoMet are used to generate spermidine in a reaction catalyzed by spermidine synthetase (SPDS) [reviewed in *72*].

In trypanosomatids, spermidine and GSH pathways intercept to generate $T(SH)_2$. The enzyme responsible for the biosynthesis of this molecule is trypanothione synthetase (TRYS), a monomeric enzyme with a molecular weight ranging from 71 kDa in *T. brucei* to 75 kDa in *C. fasciculata* [*73-76*]. This enzyme comprises two distinct catalytic domains, a C-terminal synthetase domain displaying an ATP-grasp family fold, common to C:N ligases [*77,78*], and a N-terminal amidase domain belonging to the cysteine, histidine-dependent amidohydrolase/peptidases (CHAP) superfamily [*79*]. Trypanothione synthesis is achieved by two sequential ATP-dependent conjugations of GSH molecules to spermidine, via a mono(glutathionyl)spermidine intermediate. Each conjugation step spends one ATP molecule to form a peptide bond between the carboxyl group of the glycine residue of GSH and the N^1 or the N^8 amino group of spermidine, releasing ADP and orthophosphate, trough a concerted substitution mechanism [*76*]. Importantly, the amidase domain of TRYS is capable of hydrolyzing $T(SH)_2$ to restore GSH and spermidine by a nucleophilic attack mechanism, again forming mono(glutathionyl)spermidine as intermediate. How these opposing functions are regulated to avoid cells to enter in a non-productive ATP consuming futile-cycle is still unknown. However, some suggestions have been made that point for an active role of this enzyme in the regulation of free polyamine and GSH levels under certain growth conditions [*80*].

Trypanothione synthetase is present in all members of the Trypanosomatidae family and was validated as a drug target in *T. brucei* [55,56]. However, TRYS exhibits differences within this family and sometimes even between species of the same genus. Illustrating this, *T. brucei* synthesizes $T(SH)_2$ as described above with no evidence of other substrate affinity, whereas *T. cruzi* possesses a TRYS capable of accepting spermine and other polyamines as substrate [*74*]. Moreover, it has been reported that *C. fasciculata* TRYS interacts with other enzyme, the glutathionylspermidine synthetase (GSPS) to form a heterodimer. In *C. fasciculata*, as well as in

L. infantum and *T. cruzi*, GSPS is capable of catalyzing half of the synthesis reaction of TRYS, originating the N^1/N^8-mono(glutathionyl)spermidine (GSP) intermediate as a final product [*74,80,81*].

3.4. Mono(glutathionyl)spermidine biosynthesis

Glutathionylspermidine synthetase, catalyzes the formation of mono(glutathionyl)spermidine by the ATP-consuming conjugation of one molecule of spermidine with GSH, through a mechanism similar to that of TRYS [*82*]. Like TRYS, GSPS possesses C-terminal synthetase and N-terminal amidase domains, which are capable of synthesizing mono(glutathionyl)spermidine and hydrolyzing both mono(glutathionyl)spermidine and $T(SH)_2$ to GSH and spermidine [*74,80,83,84*]. This enzyme was first found in *E. coli* as a 138 kDa homodimer [*85,86*] and latter in *C. fasciculata* as a monomeric 80 kDa protein [*80*]. The enzyme product is found in all parasites of the Trypanosomatidae family, even in species lacking GSPS. This occurrence is attributed to the fact that TRYS can generate mono(glutathionyl)spermidine either as a product of incomplete $T(SH)_2$ biosynthesis or as a hydrolysis product of $T(SH)_2$ [*74-76,80*]. Although the functional relevance of mono(glutathionyl)spermidine is not completely elucidated, it has been attributed a role in cell growth modulation, by regulating polyamines availability [*83,87*]. Additionally, there are some evidences that it can be a substrate for TR and can substitute $T(SH)_2$ in some metabolic functions, namely peroxide and ribonucleotide reduction [*57-59*].

3.5. Targeting the trypanothione system

Targeting trypanothione biosynthesis is regarded as a good strategy to fight trypanosomatids, not only because many $T(SH)_2$-dependent enzymes have been shown essential for parasite survival, as it is also known that many of the currently used trypanocidal drugs exert their effect by interfering (directly or indirectly) with the trypanothione system. This is the case of eflornithine (DFMO), used to treat sleeping sickness, which inhibits ODC in the early steps of spermidine synthesis [*88*]. Also trivalent arsenicals react directly with $T(SH)_2$, TR or tryparedoxin (TXN) leading to the death of parasites [*59*]. In addition, drugs used to treat Chagas' disease, like nifurtimox or benznidazole, are known to kill parasites by inducing oxidative stress that has to be counteracted by the trypanothione-dependent peroxidase system [*89,90*].

Finding compounds that specifically inhibit pivotal enzymes of this system, like TR or TRYS, have been the focus of many rational drug design studies. Several compounds have been reported to display good inhibitory effect against recombinant TR of different trypanosomatids [*91,92*]. However, inhibition of this protein to levels that induce parasite death have hardly been

accomplished. This fact has been attributed to the need of high inhibition levels of TR activity (95%) to impair parasite survival [*93,94*]. As for TRYS, its inhibition has already been shown to impair parasite viability at reasonable drug concentrations. Several classes of compounds have been discovered that inhibit TRYS of different trypanosomatids. Recently, paullones, a class of compounds used to treat cancer, was discovered to have a potent effect as inhibitor of TRYS [*95*]. Paullones are 7,12-dihydroindolo[3,2-*d*][1]benzazepin-6(5*H*)-ones, a class of ATP analogues known to primarily inhibit certain protein kinases, like cyclin-dependent kinases and glycogen synthetase kinase-3. Paullones synthesis was described for the first time in 1992 and since then has been applied in several medical studies from research in neurosciences to apoptosis, diabetes, cancer, embryonic development and anti-parasitic studies [*96*]. Some compounds of this family, namely the group of N^5-substituted 2-(6-oxo-6,7-dihydro-5*H*-benzo[2,3]azepino[4,5-b]indol-5-yl)-acetamides, have proven to be good inhibitors of recombinant TRYS of *C. fasciculata* (IC_{50}=30 nM) and killed *T. brucei* in culture with an LC_{50} near 2 μM (M. Comini, personal communication). Paullones interact with the ATP-binding domain of TRYS, causing irreversible inhibition, which is not counteracted by physiological concentrations of ATP or any other substrate [*95*].

4. Aims of the work

The general aim of this study was to validate the enzymatic pathway for $T(SH)_2$ biosynthesis as a drug target in *L. infantum*. As mentioned before, *L. infantum* harbors a TRYS enzyme, which catalyzes both conjugation steps required for $T(SH)_2$ synthesis, as well as GSPS, the protein that drives mono(glutathionyl)spermidine formation. So far, the essential role of *TRYS* for parasite survival has only been demonstrated for *T. brucei*, a trypanosomatid that lacks *GSPS*. In *L. infantum*, however, it cannot be taken for granted that *TRYS* is also essential, as mono(glutathionyl)spermidine, may replace some of the metabolic functions of $T(SH)_2$. Having this in mind, the specific goal of this work was to address the essential role of both *TRYS* and *GSPS* for *L. infantum* survival, by employing a reverse genetics strategy. In parallel we have also pursued the chemical validation of *TRYS* using a N^5-substituted paullone that specifically inhibits TRYS activity *in vitro*.

Chapter 2

Results

1. Analysis of *TRYS* and *GSPS* amino acid sequences

The *L. infantum* genome harbors a single copy of the *TRYS* coding sequence, which is predicted to encode a protein (*Li*TRYS) with 652 amino acids and a molecular mass of 74.23 kDa. Figure 2.1 shows the predicted amino acid sequence of *Li*TRYS and compares it to TRYS proteins of other trypanosomatids. *Li*TRYS exhibits 95.2% and 62.1% identity to the formerly characterized enzymes of *L. major* and *T. brucei*, respectively. Moreover, it shows 78.5% and 60.5 % identity to TRYS of *C. fasciculata* and *T. cruzi*, the species of trypanosomatids that, like *L. infantum*, harbor a *GSPS ORF*. Trypanothione synthetase of *L. infantum* shares with previously established TRYS a synthetase and amidase domains, and preserves the residues that were identified as being important for substrate interaction and/or binding (Figure 2.1) [*82,84,97*].

The *GSPS* of *L. infantum* is a single copy gene, which encodes a putative protein (*Li*GSPS) with 719 amino acids and a molecular mass of 80.72 kDa. This protein has 70.1% identity to the characterized GSPS of *C. fasciculata* and 49.4% identity to GSPS of *T. cruzi*. Moreover, it possesses both synthetase and amidase domains characteristic of GSPS of other organisms. Although *Li*GSPS shares only 26 % identity with the *E. coli* GSPS, it preserves the main residues that were identified as being important for interactions and/or binding of substrates. Interestingly, residues involved in homodimer formation in *Ec*GSPS are not conserved in *Li*GSPS. Instead, *Li*GSPS possesses several amino acids insertions, which, as suggested by Oza *et al.*[*82*], may be involved in GSPS interaction with TRYS. Such additional residues include a 10 amino acid insertion in the amidase domain, as well as two insertions (of 14 and 39 amino acids) in the synthetase domain (Figure 2.2). Characterization of both *Li*GSPS and *Li*TRYS was not carried out in this work, thus the possibility of heterodimer formation by these two enzymes remains unexploited in *L. infantum*.

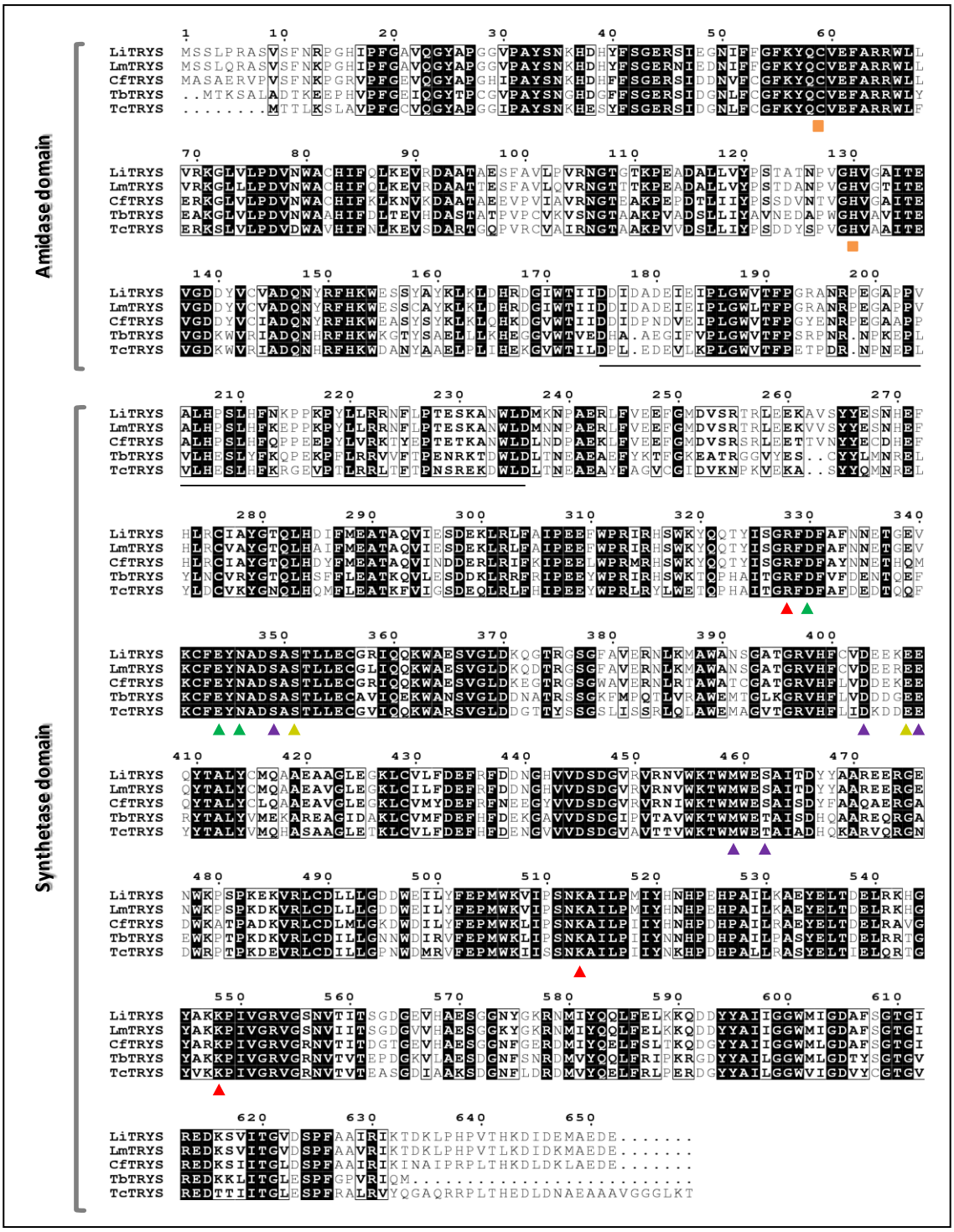

Figure 2.1. Alignment of the predicted amino acid TRYS sequences of different trypanosomatids. Protein sequence of TRYS of *L. infantum* (*Li*TRYS, accession number A4I2Z3), *L. major* (*Lm*TRYS, accession number AJ311570), *C. fasciculata* (*Cf*TRYS, accession number AF006615), *T. brucei* (*Tb*TRYS, accession number Q586P2) and *T. cruzi* (*Tc*TRYS, accession number AF311782) are represented. The amidase and synthetase domains are indicated on the left side. Conserved and similar residues are highlighted in black background and black frames, respectively. Residues of the linker region between domains are underlined. Residues involved in substrates interaction and/or binding are indicated by arrow heads: ▲- ATP, ▲- Mg^{2+}, ▲- GSH and ▲-spermidine. Residues involved in amidase activity are indicated with ■.

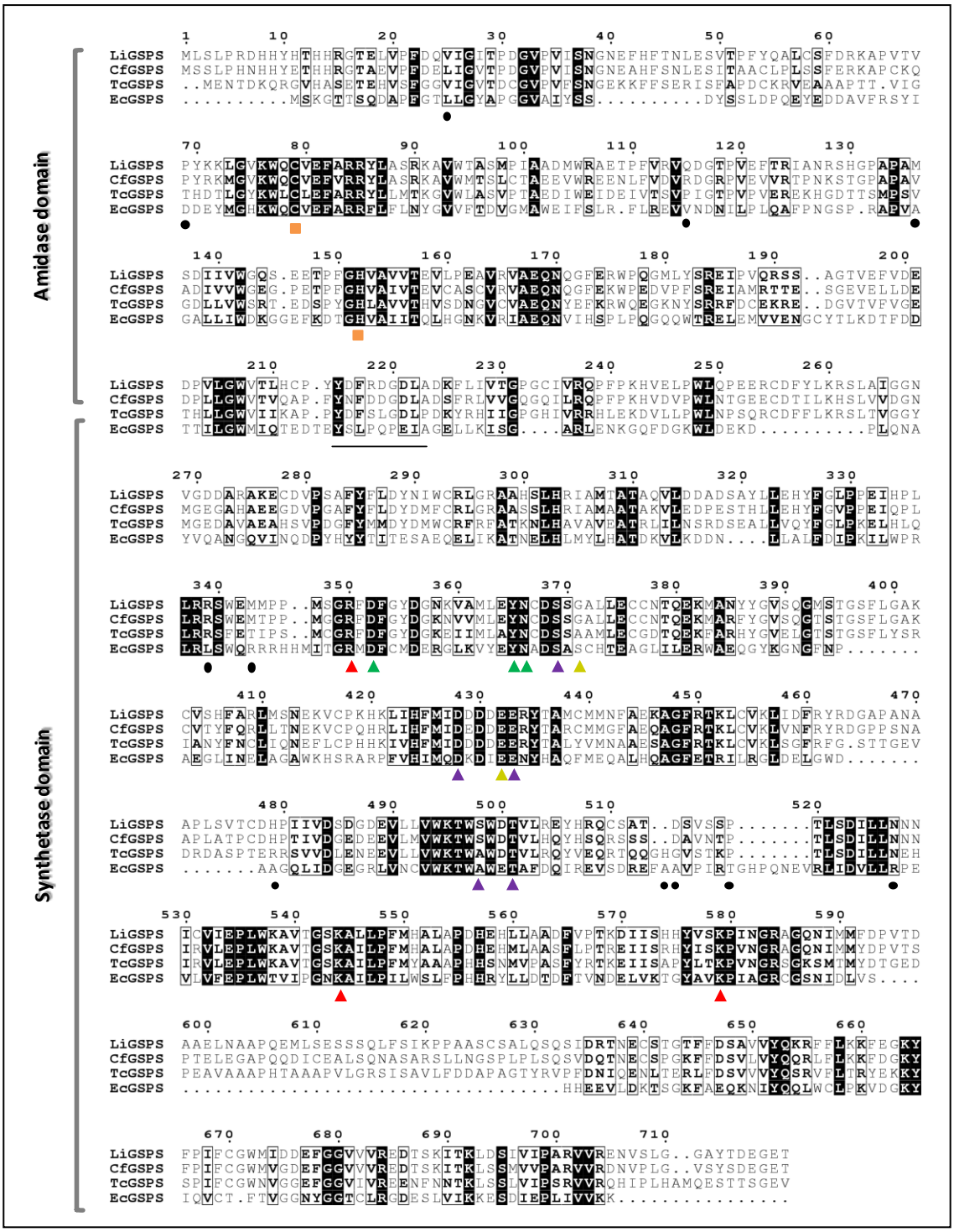

Figure 2.2. Alignment of the predicted amino acid GSPS sequences for different organisms. Protein sequence of GSPS of *L. infantum* (*Li*GSPS, accession number A4I1T8), *C. fasciculata* (*Cf*GSPS, accession number U66520), *T. cruzi* (*Tc*GSPS, accession number AY828997) and *E. coli* (*Ec*GSPS, accession number U23148) are represented. The amidase and synthetase domains are indicated on the left side. Conserved and similar residues are highlighted in black background and black frames, respectively. Residues of the linker region between domains are underlined. Residues involved in substrates interaction and/or binding are indicated by arrow heads: ▲- ATP, ▲- Mg^{2+}, ▲- GSH and ▲-spermidine. Residues involved in amidase activity are indicated with ■. Residues involved in *Ec*GSPS monomer-monomer interaction are indicated by ●.

2. Expression of *TRYS* along the *L. infantum* life cycle

Expression of TRYS was investigated by western blot throughout the life cycle of *L. infantum*, including promastigotes and axenically grown amastigotes. Results, depicted in Figure 2.3, show that the enzyme is expressed as a peptide of approximately 75.5 kDa in both life cycle stages of the parasite. During promastigote growth expression of TRYS gradually increases from the early logarithmic phase to the stationary phase.

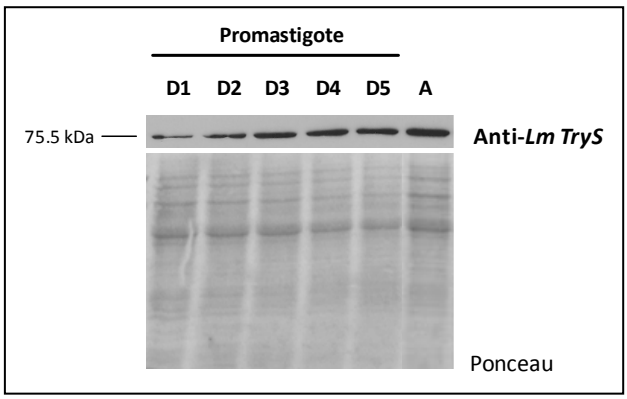

Figure 2.3. Expression of TRYS during the *L. infantum* life cycle. Western blot analysis of total protein extracts of *L. infantum*, incubated with an antibody directed against the *L. major* TRYS. Protein extracts from early logarithmic (day 1 of culture or D1) to stationary (day 5 of culture or D5) promastigotes were analyzed, as well as from axenically grown amastigotes (A). Ponceau staining of the western blot membrane is shown as a control.

3. Genetic validation of *TRYS*

Essentiality of *TRYS* was addressed following a gene replacement strategy. Since *L. infantum* is a diploid organism, two rounds of gene targeting were required to target both *TRYS* alleles. Accordingly, two *TRYS* replacement constructs were generated. To that end, part of the non-coding sequences flanking the *TRYS ORF* were cloned into two different plasmids, carrying either the hygromycin B phosphotransferase gene (*HYG*), or the phleomycin hydrolase gene (*PHLEO*), which confer resistance to hygromycin B and phleomycin, respectively (Figure 2.4). Prior to transfection, these constructs were linearized (by *Hind*III/*Sac*I double restriction) and the replacement cassettes separated from the plasmids backbone.

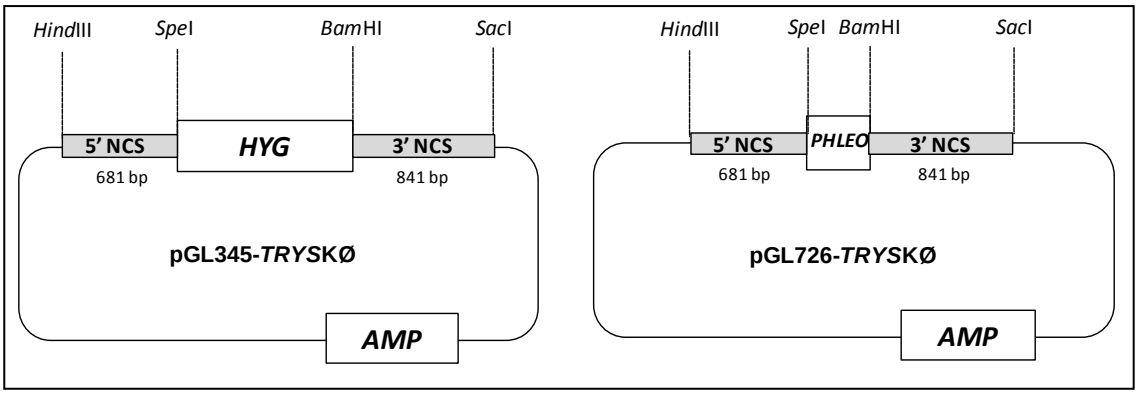

Figure 2.4. Schematic representation of *TRYS* replacement constructs. The constructs used to target *TRYS* (pGL345-*TRYS*KØ and pGL726-*TRYS*KØ) are represented. Hygromycin B phosphotransferase (*HYG*), phleomycin hydrolase (*PHLEO*), beta-lactamase (*AMP*) *ORF*s and 5', 3' non-coding sequences (5'NCS and 3'NCS) flanking *TRYS ORF* are represented in boxes. Restriction sites used to assemble the constructs are also shown.

3.1. Generation of heterozygous Δtrys::HYG/TRYS L. infantum parasites

The first *TRYS* allele was targeted with the *HYG* replacement cassette. Following the transfection procedure, hygromycin-resistant promastigotes were selected and individual clones tested by PCR using primers P8 and P9 (see Table 3.1 in the "Material & Methods" section). Primer P8 is a sense primer that anneals in the gene upstream of *TRYS* and was designed to diagnose for the correct integration of disruption constructs in the *TRYS locus*, whereas P9 is an anti-sense primer designed to anneal at the beginning of *HYG ORF* (Figure 2.5 A). Analysis of three clones testing positive for the correct integration of the *HYG* cassette in the *TRYS locus* is shown in Figure 2.5 B. These heterozygous Δ*trys*::*HYG/TRYS* mutants (hereafter designated as *trys*$^{+/-}$) presented no differences regarding their morphology (not shown) and growth rate (Figure 2.5 C) when compared with wildtype parasites, indicating that one *TRYS* copy is sufficient to sustain promastigote growth *in vitro*.

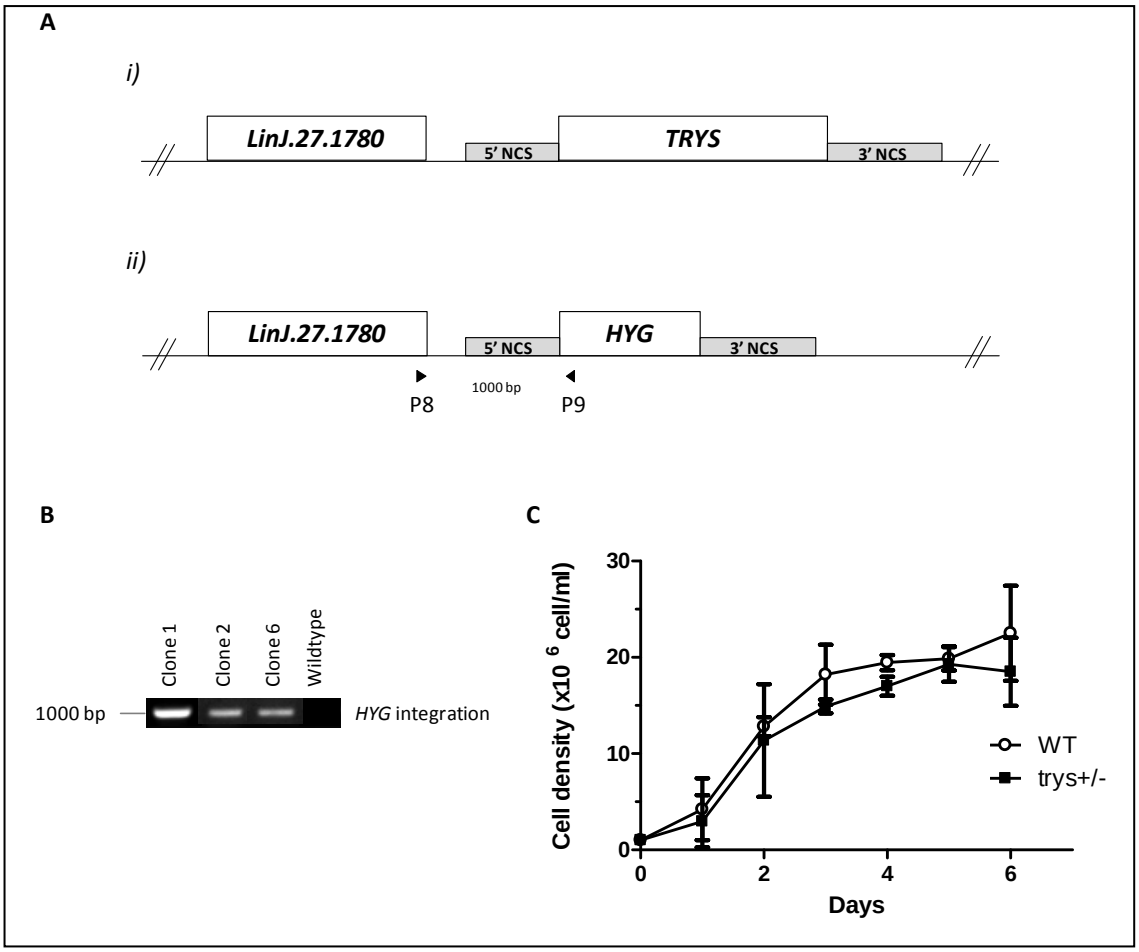

Figure 2.5. Generation of *L. infantum* heterozygous *trys*$^{+/-}$ promastigotes. (A) Representation of the *L. infantum TRYS locus* with its *LinJ.27.1780* upstream gene in *trys*$^{+/-}$ parasites. Both *i)* wildtype and *ii) HYG*-targeted alleles are shown. The 5' and 3' NCS used to construct the *HYG* replacement cassette are represented in grey boxes. Primers P8 and P9 used in the PCR diagnosis for the correct integration of the *HYG* cassette in the *TRYS locus* are indicated by arrowheads. The number in between arrowheads refers to the expected size of the corresponding PCR product. **(B)** PCR analysis of genomic DNA from three hygromycin-resistant mutants (clones 1, 2 and 6) and wildtype parasites, using primers P8 and P9. **(C)** Growth curves of wildtype (WT) and *trys*$^{+/-}$ (clone 1) promastigotes. Parasites were seeded at 10^6 at day 0 and counted daily with a hemocytometer. Data represent the mean and standard deviation of two independent growth curves.

3.2. Attempts to generate L. infantum TRYS null mutants

In order to replace the second *TRYS* allele, the *trys*$^{+/-}$ clone 1 was selected for transfection with the *PHLEO* replacement cassette. Following the transfection procedure, parasites resistant to both hygromycin and phleomycin were selected and three individual clones analyzed by PCR to diagnose for different events, namely (see Figure 2.6 A): *i)* integration of the *PHLEO* cassette into the *TRYS locus*; *ii)* presence of the *PHLEO, HYG* and *TRYS ORFs*; *iii)* maintenance of the *HYG* cassette in its correct position and *iv)* configuration of the endogenous *TRYS locus*. The results, depicted in Figure 2.6 B, show that none of the clones tested positive for the correct integration of the *PHLEO* cassette in the *TRYS locus*. Still, the *PHLEO ORF* was

amplified, at least in clones 3 and 5. The *HYG ORF* was kept in its correct location in the *TRYS locus* during the second transfection procedure. Importantly, all three clones retained the *TRYS ORF*, in its original *locus* (clones 3 and 5) or possibly in other position of the genome (clone 1). The failure to eliminate both gene alleles of *TRYS* is *per se* suggestive that this may be an essential gene [*31*].

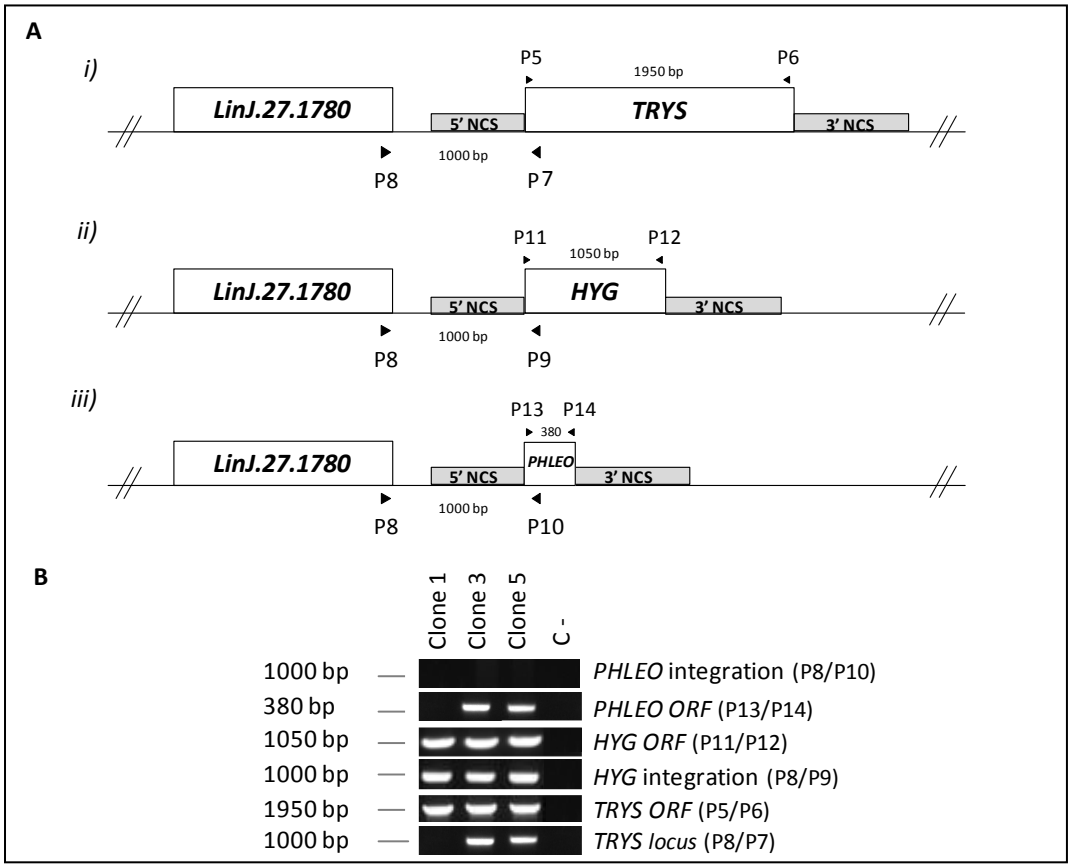

Figure 2.6. *Leishmania infantum* parasites obtained upon the second round transfection with the *PHLEO* replacement cassette. (A) Representation of the *L. infantum TRYS locus* with its *LinJ.27.1780* upstream gene in *i)* the wildtype allele and as expected upon the recombination events with the *ii) HYG* and *iii) PHLEO* integration cassetes. The 5' and 3' NCS used to construct the *HYG* and *PHLEO* replacement cassettes are represented in grey boxes. Primers used in the PCR diagnosis for the different integration events in the *TRYS locus*, as well as to amplify the *TRYS*, *HYG* and *PHLEO ORF*s are indicated by arrowheads. Numbers in between arrowheads refer to the expected size of the corresponding PCR products. **(B)** PCR analysis of genomic DNA from three hygromycin- and phleomycin-resistant mutants (clones 1, 3 and 5). Primers used in the PCR reactions are indicated in parenthesis. The same reactions were carried out in the absence of DNA to serve as negative control (C -).

3.3. Generation of L. infantum TRYS null mutants requires previous complementation with episomic TRYS

Following two sequential rounds of transfection we were not able to eliminate both *TRYS* alleles, suggesting that this gene might be essential to promastigote survival. To rule out the possibility that technical limitations could be hindering the isolation of *TRYS*-depleted

parasites, we followed another experimental approach. This consisted in introducing an episomal copy of the *TRYS* gene into *trys*$^{+/-}$ mutants prior to transfection with the *PHLEO* replacement cassette. To that end the *trys*$^{+/-}$ clone 1 was transfected with the trypanosomatidal expression vector pTEX-*NEO* carrying the *TRYS ORF* (Figure 2.7 A). This vector carries the neomycin phosphotransferase gene (*NEO*), which confers resistence to geneticin. Following the transfection procedure, three hygromycin- and geneticin-resistant clones were confirmed by PCR (Figure 2.7 B) to carry an episomal copy of *TRYS* (P25/P6), as well as to preserve the *HYG ORF* in its the correct location in the *TRYS locus* (P8/P9). One of the clones (clone 8) was further tested for the integrity of the episome using different combinations of primers (P5/P10 and P23/P26). The results, depicted in Figure 2.7 B, indicated that the pTEX-*NEO-TRYS* episome was intact in this clone. These *trys*$^{+/-}$ parasites carrying an extrachromosomal copy of *TRYS* were subsequently used for the second round of targeting of the *TRYS locus* with the *PHLEO* cassette. The outcome of this transfection was the isolation of parasites resistant to hygromycin, geneticin and phleomycin. Southern blot analysis of three of these clones confirmed the successful replacement of both *TRYS* alleles. Illustrating this, the 4.9 kb band, which corresponds to the chromosomal *TRYS* copy in *Sac*II-digested DNA (Figure 2.8 A), was not detected in any of these mutants (Figure 2.8 B). Instead, a 7.6 kb band, matching the episomal *TRYS* copy, was recognized by the *TRYS* probe. This 7.6 kb fragment also hybridized to the *NEO* probe, further confirming the presence of the *TRYS* episome. Integrity of both *HYG*- and *PHLEO*-targeted alleles was verified using the corresponding radiolabeled-*ORF*s as probes. As shown in Figure 2.8 B, the *HYG* probe labeled one band of approximately 2 kb, which likely contains the 1.9 and 2.1 kb fragments that result from *Sac*II restriction of the *HYG* allele (Figure 2.8 A). As for the *PHLEO* probe, it detected a 3.3 kb band, as expected for the correct integration of the *PHLEO* cassette into the *TRYS locus*. These Δ*trys*::*HYG*/Δ*trys*::*PHLEO* [pTEX-*NEO-TRYS*] mutants will be from here on be designated as *trys*$^{-/-}$/+*TRYS*.

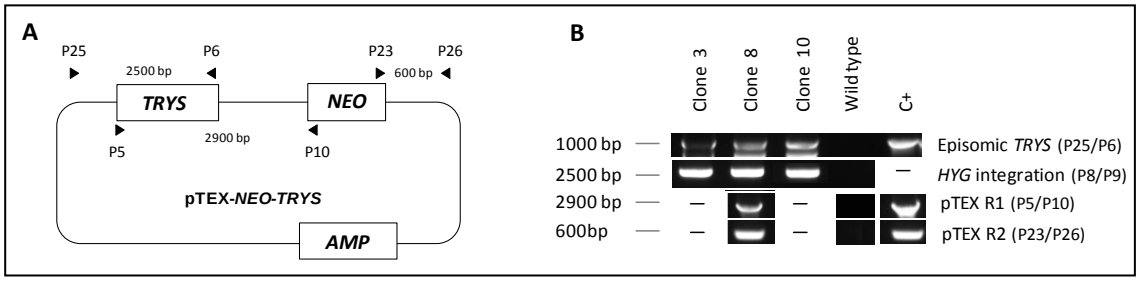

Figure 2.7. Analysis of pTEX-*NEO-TRYS* complemented *trys*$^{+/-}$ mutants. (A) Schematic representation of the pTEX-*NEO-TRYS* plasmid. Trypanothione synthetase (*TRYS*), neomycin phosphotransferase (*NEO*) and beta-lactamase (*AMP*) *ORF*s are represented in boxes. Primers used in the PCR analysis in (B) are indicated by arrowheads. Numbers in between arrowheads refer to the expected size of the corresponding PCR products. **(B)** PCR analysis of genomic DNA from three independent pTEX-*NEO-TRYS* complemented *trys*$^{+/-}$ mutants (clones 3, 8 and 10) and wildtype promastigotes. Purified pTEX-*NEO-TRYS* plasmid was used as template to serve as positive control in some of the reactions (C +). Primers used in the PCR reactions are indicated in parenthesis and their location is represented in (A), except for primers P8 and P9, whose binding site is depicted in Figure 2.6.

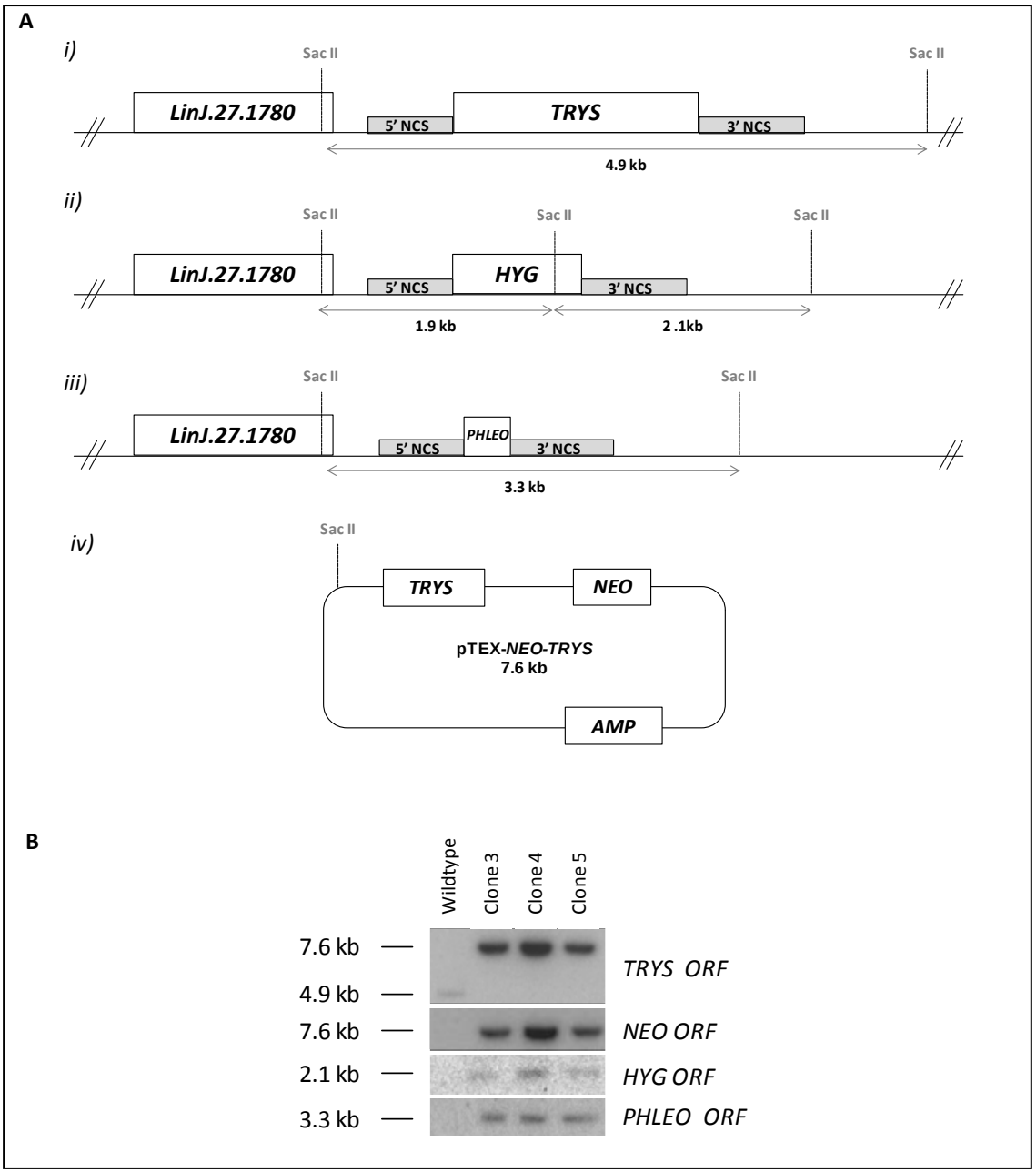

Figure 2.8. Southern blot analysis of *trys*$^{-/-}$/+*TRYS* mutants. (A) Representation of the *L. infantum TRYS locus* with its *LinJ.27.1780* upstream gene *i)* before and after *TRYS* replecement by the *ii) HYG* and *iii) PHLEO* cassettes. *iv)* Also depicted is the scheme of the pTEX-*NEO-TRYS* episome. Sizes of the predicted SacII-restriction fragments are indicated. **(B)** Southern blot of *Sac*II-digested genomic DNA of wildtype and three *trys*$^{-/-}$/+*TRYS* mutants (clones 3, 4 and 5) hybridized with radiolabeled *NEO*, *TRYS*, *HYG* and *PHLEO ORF*s.

3.4 Maintenance of the pTEX-NEO-TRYS episome in the absence of drug pressure confirms gene essentiality in promastigotes

Leishmania episomes are highly unstable and are usually lost over time unless they provide an advantage to the parasite. The pTEX-*NEO* vector used to introduce the *TRYS* episomal copy is no exception to this and, as such, it is lost when parasites are cultured in the

absence of geneticin [*98*]. To otain definitive proof of *TRYS* essentiality, we used this feature of pTEX-*NEO* and cultured *trys*$^{-/-}$/+*TRYS* promastigotes for 6 months in absence of geneticin. The maintenance of the vector was tested in two independent clones by Southern blot and by a geneticin-resistance assay.

Southern blot analysis revealed that both clones retained the pTEX-*NEO*-*TRYS* plasmid. As depicted in Figure 2.9 A, hybridization of *Sac*II-digested genomic DNAs of the *trys*$^{-/-}$/+*TRYS* mutants with either the radiolabeled *NEO* or *TRYS ORF*s yielded a band of approximately 7.6 kb, corresponding to the pTEX-*NEO*-*TRYS* plasmid. Control for the lost of plasmid was performed in parallel using wildtype parasites carrying the empty pTEX-*NEO* vector. Three months in the absence of geneticin were enough for these parasites to lose the plasmid, as evidenced by disappearance of the 5.6 kb *Sac*II-fragments hybridized to the *NEO* probe in parasites cultured in the absence of the drug.

Results obtained in the geneticin-resistance assays were in line with the Southern blot analysis. As shown in Figure 2.9 B, control parasites cultured in the absence of G418 had lost resistance to this drug, contrary to control parasites that were cultured in parallel in the presence of drug pressure. Both *trys*$^{-/-}$/+*TRYS* clones cultured in the absence of G418 were resistant to this drug, indicating that they preserved the pTEX-*NEO*-*TRYS* plasmid.

Altogether the results presented here show that in the absence of drug pressure the episomal copy of *TRYS* is not lost in parasites lacking the endogenous gene. Requirement for *TRYS* likely provides the pressure to maintain the episome, thus implying that this gene is essential for promastigote survival.

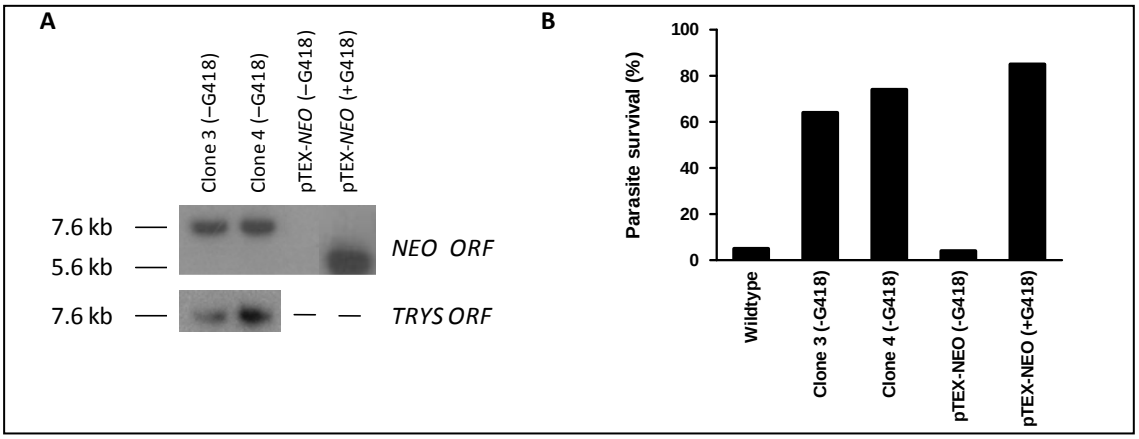

Figure 2.9. Southern blot analysis of *trys*$^{-/-}$/+*TRYS* mutants cultured in the absence of geneticin. (A) Southern blot of *Sac*II-digested genomic DNA of two *trys*$^{-/-}$/+*TRYS* mutants (Clones 3 and 4) cultured for 6 months in the absence of geneticin and of control parasites (pTEX-*NEO*) cultured for 3 months either in the absence (-G418) or in the presence of 40 µg ml^{-1} of geneticin (+G418). Blots were hybridized with radiolabeled *NEO* and *TRYS ORF*s. **(B)** Effect of geneticin on the survival of *trys*$^{-/-}$/+*TRYS* mutants (clones 3 and 4) that had been cultured for 6 months in the absence of drug pressure. Control parasites are wildtype (WT) and pTEX-*NEO* transfectants [grown for 3 months either in the absence (-G418) or presence (+G418) of geneticin]. Four to seven days after the addition of G418, the number of promastigotes was counted with a hemocytometer and the data expressed as the percentage of parasite number in cultures to which no drug had been added. Data refers to one experience.

3.6. Assessment of TRYS essentiality in the amastigote stage of L. infantum

For a gene to be validated as a drug target, it has to be proven essential in the mammalian stage of the parasite. To assess *TRYS* essentiality in amastigotes, BALB/c mice were inoculated with three *trys*$^{+/-}$/+*TRYS* clones and with pTEX-*NEO* control parasites. Nine weeks after infection, control parasites were recovered from the liver and spleen of infected mice and analyzed for the presence of the pTEX-*NEO* plasmid by PCR and by a geneticin-resistance assay. Both experimental approaches revealed that these parasites retained the plasmid upon 9 weeks in mice. As such, analysis of the *trys*$^{+/-}$/+*TRYS* clones was not performed at this time point and will have to await at least 4 additional weeks, as to guarantee loss of pTEX-*NEO* by the control parasites [*67*].

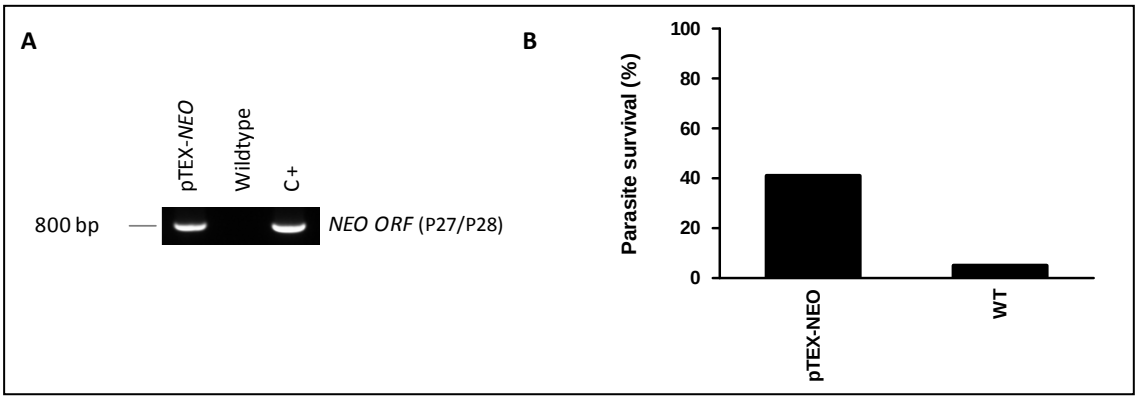

Figure 2.10. Analysis of pTEX-*NEO* transfected parasites recovered from mice 9 weeks after infection. (A) PCR analysis of genomic DNA from control parasites (pTEX-*NEO*) recovered from the liver and spleen of BALB/c mice 9 weeks after infection. Purified pTEX-*NEO*-*TRYS* plasmid was used as template in the positive control reaction (C +). Primers used in the PCR reactions are indicated in parenthesis. **(B)** Effect of geneticin on the survival of control parasites (pTEX-*NEO*) recovered from the liver and spleen of BALB/c mice 9 weeks post infection. Four days after the addition of G418, the number of promastigotes was counted with a hemocytometer and the data expressed as the percentage of parasite number in cultures to which no drug had been added. Data represent counts from one experience. Wildtype parasites were used as control in both (A) and (B).

4. Chemical validation of *TRYS*

4.1. Preliminary assays

To chemically validate a gene, one must first identify compounds that specifically inhibit the product of that gene. The N^5-substituted paullones were identified by our German partner (at MOLISA, GmbH) as potent inhibitors of purified recombinant *L. infantum* TRYS with IC_{50} values in the nanomolar range (Table 2.1). These compounds were therefore chosen to carry out the chemical validation of *Li*TRYS.

From the existing set of compounds, we chose four that exhibited the highest *Li*TRYS inhibitory effect *in vitro*, namely FS-304, FS-554, FS-528 and FS-99a. The leishmanicidal effect of these compounds was tested at 5 and 50 µM against intracellular *L. infantum*

amastigotes residing in monolayers of murine peritoneal macrophages. Upon incubation with drugs, cells were stained for immunofluorescence analysis. Micrographs representative of this experiment are shown in Figure 2.11. As revealed from alterations on cell morphology, three of these drugs (FS-304, FS-528 and FS-99a) were toxic to macrophages at 50 µM. Moreover, at the other concentration tested (5 µM), these compounds had no apparent anti-parasitic effect, as indicated by the high load of intracellular amastigotes. On the contrary, FS-554 did not significantly affected macrophages morphology at 50 µM and reduced the number of intracellular amastigotes at both concentrations tested (Figure 2.11).

Before quantifying the effect of FS-554 on amastigote survival, we performed a viability assay to access toxicity of this drug towards macrophages. To that end, non-infected macrophages were incubated with increasing concentrations of FS-554 and their viability measured by the resazurin assay. Results showed a marked decrease in cell viability for concentrations above 30 µM (1.5 log units in Figure 2.12 A). As such, the anti-parasitic effect of the compound was only assessed at 5 µM, although the microscopic observation of the monolayers at 50 µM indicates a near 100 % reduction of infection (Figure 2.11). Quantification of the leishmanicidal effect of FS-554 at 5 µM revealed a 2.25 fold reduction in the infection index (Figure 2.12 B).

To discard the possibility that the observed leishmanicidal effect of FS-554 could result from activation of macrophages, we employed the Griess assay to measure the amount of nitrite (NO_2^-), a microbicidal molecule that is generated by activated macrophages [*10*]. Formation of NO_2^- was measured in the supernatant of infected and non-infected macrophages cultured in the presence of increasing amounts of FS-554. Results revealed that, in all tested conditions, the amount of NO_2^- was undetectable, thus indicating that the anti-parasitic effect of FS-554 was not the consequence of macrophage activation (data not shown).

Altogether, these results indicate that FS-554 is an appropriate compound to be used in the chemical validation of *TRYS* in both *L. infantum* life cycle stages.

Table 2.1. List of N^5-substituted paullones and their IC_{50} values against purified recombinant *L. infantum* TRYS.

Code	IC_{50} (nM)
FS-304	31,2
FS-554	52
FS-528	208
FS-99a	235
FS-202	295
FS-586	319
FS-573	469
FS-185	537
FS-634	785
FS-151	837
FS-628	951

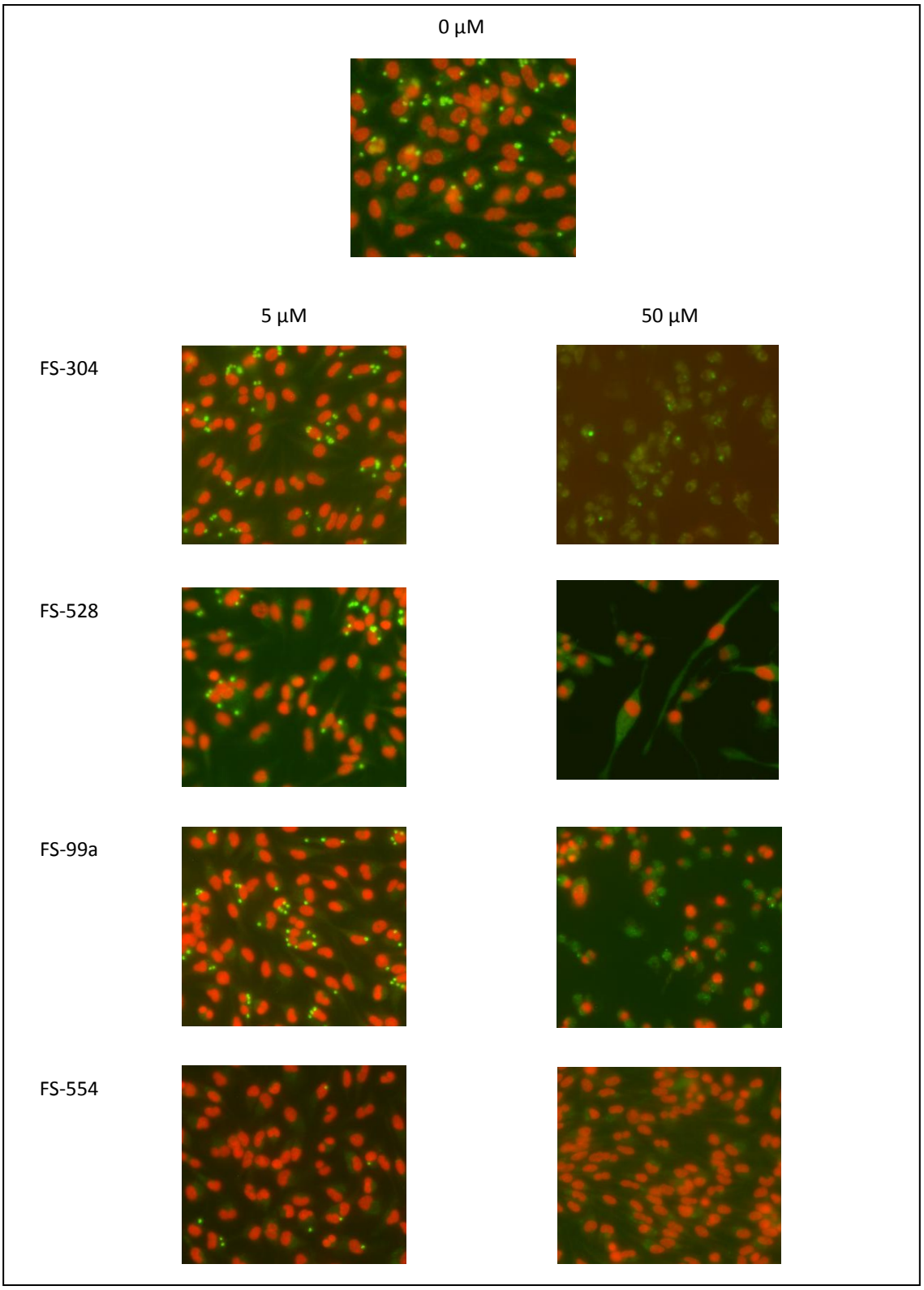

Figure 2.11. Preliminary screening of N^5-substituted paullones on intracellular *L. infantum* amastigotes. Representative IFAT micrographs of each tested compound at 5 and 50 µM. Nucleus of macrophages are marked with DAPI (shown in red) and amastigotes are labeled with primary antibodies anti-*Lic*TXNPx1 and anti-*Li*mTXNPx (shown in green).

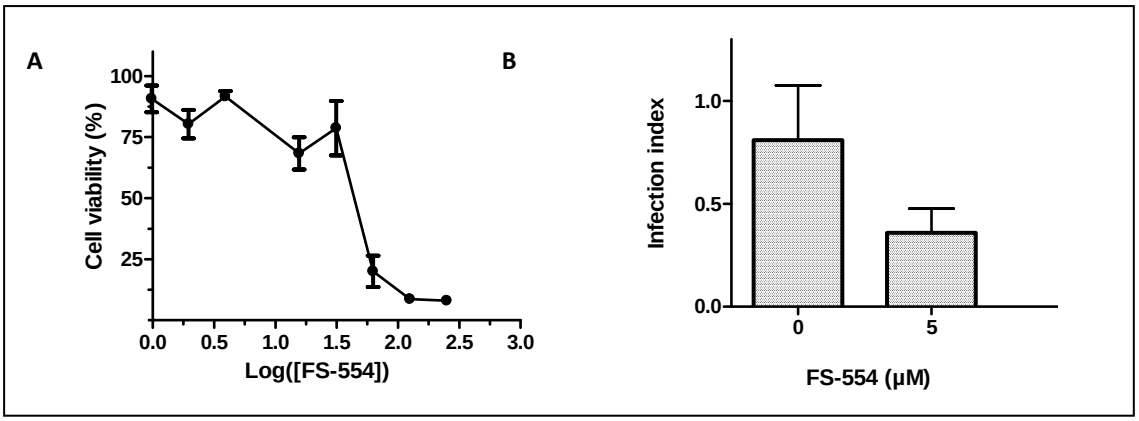

Figure 2.12. Cytotoxic effect of FS-554 on macrophages and intramacrophagic *L. infantum* amastigotes. (A) Effect of FS-554 on the viability of murine peritoneal macrophages. Macrophages were incubated with different concentrations of FS-554 (0 to 250 µM) and viability addressed by the resazurin assay. Data are expressed as a percentage of cell viability relative to macrophages to which no FS-554 was added. **(B)** Effect of FS-554 against intracellular amastigotes. Infection index of monolayers of *L. infantum*-parasitized murine peritoneal macrophages upon treatment with 5 µM FS-554 for 48 hours. Data in (A) and (B) represent means and standard deviations of triplicate measurements from one experience.

4.2. TRYS expression levels correlate with resistance of L. infantum to FS-554

Chemical validation of TRYS was addressed by evaluating the potency of FS-554 against parasites expressing different levels of the enzyme. To that end, we made use of the previously produced heterozygous *trys*$^{+/-}$ mutants, as low TRYS-expressing parasites, and addionally transfected wildtype promastigotes with the pTEX-*NEO-TRYS* plasmid to obtain TRYS overexpressers. Correct genotypes of these transfectants were confirmed by Southern blot (Figure 2.13 A). The expression levels of TRYS were kept high by addition of geneticin to the cultures, which guarantees the maintenance of several copies of the plasmid, hence of episomic *TRYS*. Wildtype parasites transfected with pTEX-*NEO* were used as a control of the TRYS overexpressers, in order to evade effects of geneticin in the parasite growth. Of notice, the TRYS overexpressers did not show any differences regarding morphology (not shown) and growth rate (Figure 2.13 B) with respect to control parasites.

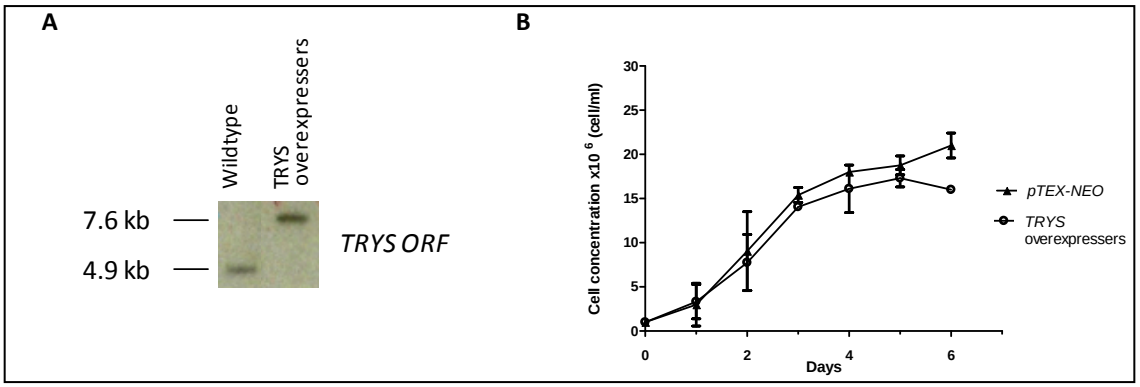

Figure 2.13. Analysis of TRYS overexpressers. (A) Southern blot analysis of TRYS overexpressers parasites. Hybridization of *Sac*II-digested DNA from TRYS overexpressers with the radiolabeled *TRYS ORF*, yielded a 7.6 kb band corresponding to the pTEX-*NEO*-*TRYS* plasmid. Please notice that the 4.9 kb band corresponding to the endogenous *TRYS locus* is not detected in these mutants, possibly due to technical limitations. **(B)** Growth curves of control parasites (pTEX-*NEO*) and TRYS overexpressers in the presence of 40 µg ml^{-1} geneticin. Promastigotes were seeded at 10^6 at day 0 and counted daily with a hemocytometer. Data represent means and standard deviations of two independent growth curves.

The leishmanicidal effect of FS-554 was subsquently tested towards promastigotes expressing different levels of TRYS. Relative amounts of TRYS in wildtype, *trys*$^{+/-}$, TRYS overexpressers and control parasites were verified by western blot (Figure 2.14 A). The results, depicted in Figure 2.14 B, show that parasites with decreased TRYS expression are more sensitive to FS-554, while overexpression of the protein confers promastigotes resistance to this drug. Non-linear fit of this data was performed, allowing us to determine the EC_{50} of FS-554 towards each parasite line (Table 2.2).

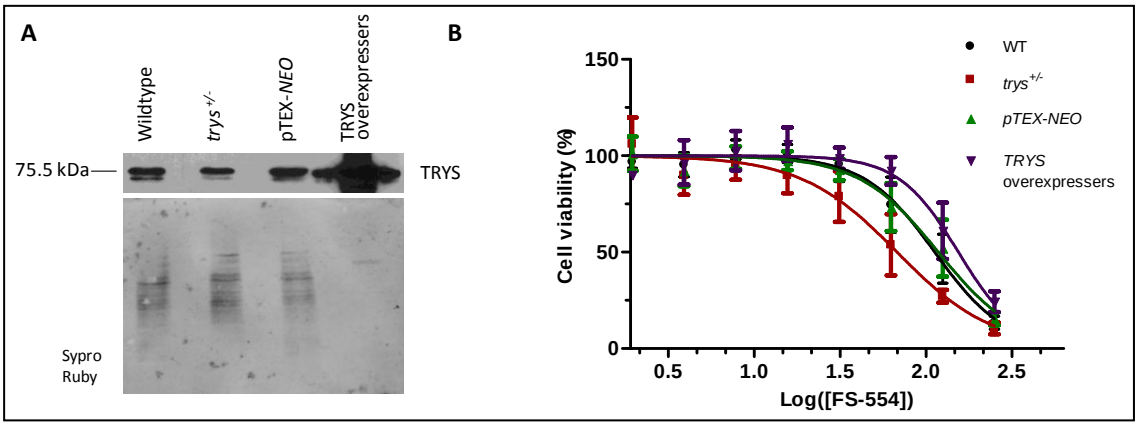

Figure 2.14. Correlation between TRYS expression levels and susceptibility of *L. infantum* promastigotes to FS-554. (A) Western blot analysis of total protein extracts from previous synchornized cultures of wildtype, homozygous *trys*$^{+/-}$, pTEX-*NEO* and TRYS overexpressers, incubated with an antibody directed against the *L. major* TRYS. Sypro Ruby® staining of the western blot membrane is shown as a control. **(B)** The same parasite lines were tested for their viability upon incubation with increasing concentrations of FS-554 (0 to 250 µM). Promastigotes were seeded at 10^6 cell ml^{-1} and incubated with the drug for 48 h. Cell viability was determined by the resazurin assay after 24-96 h upon the addition of resazurin. Data represent means and standard deviations of triplicate measurements from three independent experiences. Curves are the non-linear fit of the data, obtained using a two-parameter EC_{50} equation provided by Graphpad prism 5 software.

Table 2.2. EC_{50} values of FS-554 against promastigotes expressing different levels of TRYS.

	Wildtype	*trys*$^{+/-}$	pTEX-*NEO*	TRYS Overexpressers
EC_{50} (µM)	112.3 ± 1.1	67.77 ± 1.2	117,7 ± 1.1	154,1 ± 1.1

The relative susceptibility of wildtype and *trys*$^{+/-}$ mutants to FS-554 was also addressed in intramacrophagic amastigotes. In line with the results obtained with promastigotes, *trys*$^{+/-}$ were more susceptible to FS-554 than wildtype parasites, as deduced from the infection indexes of macrophages treated with this drug. As depicted in Figure 2.15, at 15 µM FS-554 the infection index of trys$^{+/-}$ parasites decreased 1.5 fold, while having no effect on wildtype cells. Likewise, at 30 µM the effect of FS-554 was also more pronounced towards *trys*$^{+/-}$ (a 2.5 fold reduction in infection index), than towards wildtype *L. infantum* amastigotes (1.7 fold reduction in infection index).

Together these results strongly suggest that the *L. infantum* TRYS is targeted by FS-554 in both the promastigote and the amastigote stages, in other words, that it is prone to inactivation with drug-like compounds.

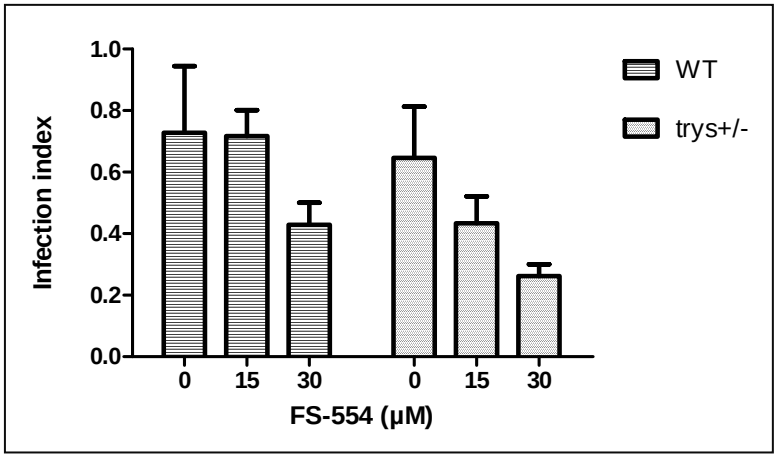

Figure 2.15. Effect of FS-554 on survival of intramacrophagic *L. infantum* amastigotes expressing different levels of TRYS. Infection indexes of wildtype (WT) and heterozygous *trys*$^{+/-}$ amastigotes at 0, 15 and 30 µM of FS-554. Data represent means and standard deviations of triplicate measurements from one experience.

5. *GSPS* functional characterization

Functional characterization of *GSPS* was performed following the same rationale described for *TRYS* genetic validation. Accordingly, two replacement constructs were generated by cloning part of the 5' NCS and 3'NCS flanking the *GSPS ORF* into two different plasmids, carrying either the blasticidin S deaminase (*BSD*) gene or the puromycin N-acetyl-tranferase (*PAC*) gene, which confer resistance to blasticidin or puromycin, respectively. Prior to

transfection, these constructs were linearized (by *Hind*III/*Bgl*II double restriction) and the plasmid backbone rejected (Figure 2.16).

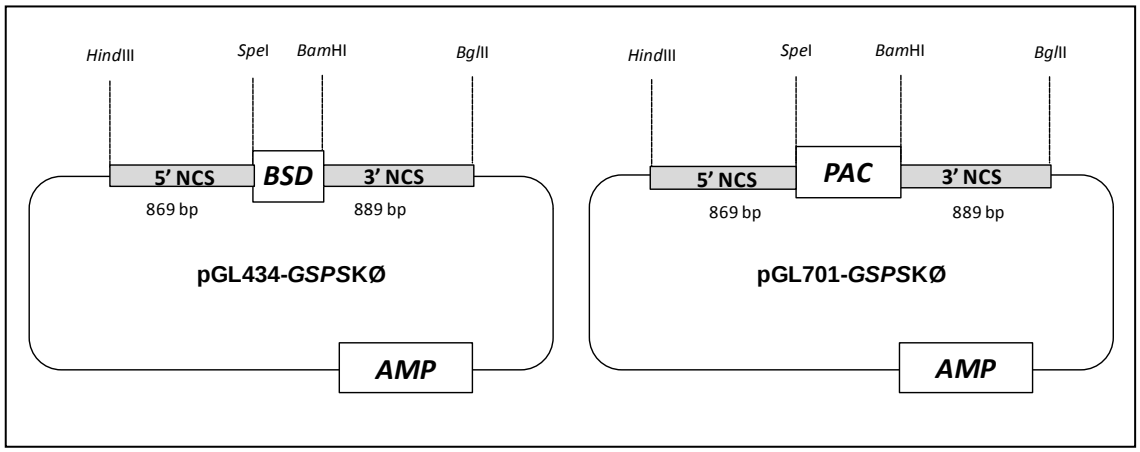

Figure 2.16. Schematic representation of *GSPS* replacement constructs. Constructs used to target *GSPS* (pGL434-*GSPS*KØ and pGL701-*GSPS*SKØ), are represented. Blasticidin S deaminase (*BSD*), puromycin N-acetyl-tranferase (*PAC*), beta-lactamase (*AMP*) *ORF*s and 5' and 3' non-coding sequences (5'NCS and 3'NCS) flanking the *GSPS ORF* are represented in boxes. Restriction sites used to assemble constructs are also shown.

5.1. Generation of L. infantum heterozygous Δgsps::BSD/GSPS parasites

In order to obtain heterozygous *GSPS* parasites, the first *GSPS* allele was targeted with the *BSD* replacement cassette. Following the transfection procedure, blasticidin-resistant promastigotes were selected and individual clones tested by PCR using primers P19, P20 and P22 (see Table 3.1 in "Matherial & Methods"). Primer P19 is a sense primer designed to anneal in the gene upstream of *GSPS* (*LinJ.25.2490*), thus allowing the diagnosis for the correct integration of the disruption constructs in the *GSPS locus*. Primers P20 and P22 are anti-sense primers designed to anneal at the beginning of the *BSD* and the *GSPS ORF*s, respectively (Figure 2.17 A). The result of the PCR analysis of one clone testing positive for the correct integration of the *BSD* cassette in the *GSPS locus*, as well as for the maintainance of one *GSPS* allele, is shown in Figure 2.17 B. This heterozygous *Δgsps::BSD/GSPS* mutant (hereafter designated as $gsps^{+/-}$) presented no morphologic alterations in comparison to wildtype promastigotes (not shown).

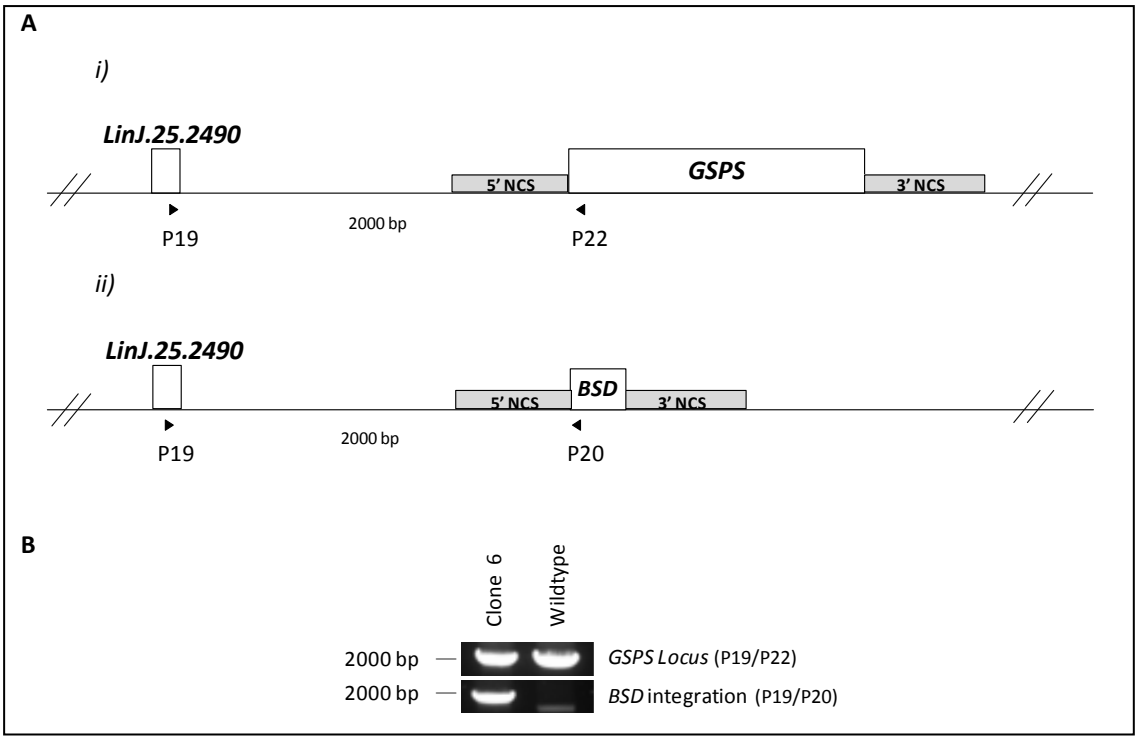

Figure 2.17. Generation of heterozygous *gsps*$^{+/-}$ promastigotes. (A) Representation of the *L. infantum GSPS locus* with its *LinJ.25.2490* upstream gene in *gsps*$^{+/-}$ parasites. Both *i)* wildtype and *ii) BSD*-targeted alleles are shown. The 5' and 3' NCS used to construct the *BSD* replacement cassette are represented in grey boxes. Primers used in PCR diagnosis for the correct integration of the *BSD* cassette in the *GSPS locus* are indicated by arrowheads. The number in between arrowheads refers to the expected size of the corresponding PCR product. **(B)** PCR analysis of genomic DNA from one blasticidin-resistant mutant (clone 6) and wildtype promastigotes, using the primers indicated in parenthesis.

5.2. Attempts to generate L. infantum GSPS null mutants

Targeting of the second *GSPS* allele was performed in the *gsps*$^{+/-}$ clone 6 with the *PAC* replacement cassette. Following the transfection procedure, parasites resistant to both blasticidin and puromycin were selected and the polyclonal culture analyzed by PCR to diagnose for different events, namely: *i)* integration of the *PAC* cassette into the *GSPS locus*; *ii)* maintenance of the *BSD* cassette in its correct position and *iii)* preservation of the endogenous *GSPS locus* (see figure 2.18 A). Results indicate that the polyclonal population tested positive for all three events. This is possibly due to the presence of genomic DNA from different clones serving as templates in the PCR reactions. Efforts are being made to isolate individual clones and to conclude about *GSPS* essentiality in *L. infantum*.

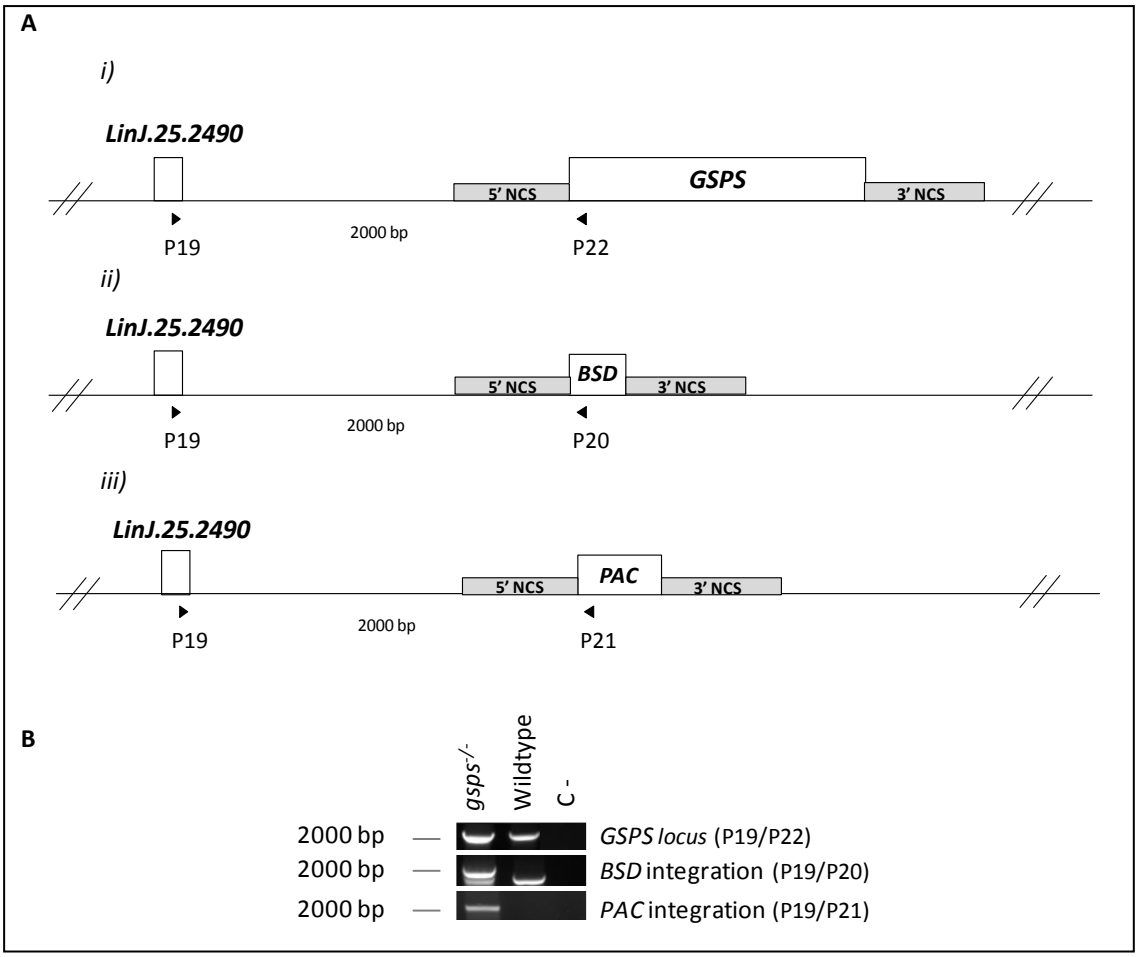

Figure 2.18. *L. infantum* parasites obtained upon the second round of transfection to target the *GSPS* locus with the *PAC* replacement cassette. (A) Representation of the *L. infantum GSPS locus* with its *LinJ.25.2490* upstream gene in *i)* the wildtype allele and as expected upon the recombination events with the *ii) BSD* and *iii) PAC* integration cassetes. The 5' and 3' NCS used to construct the *BSD* and *PAC* replacement cassettes are represented in grey boxes. Primers used in the PCR diagnosis for the different integration events in the *GSPS locus* are indicated by arrowheads. Numbers in between arrowheads refer to the expected size of the corresponding PCR products. **(B)** PCR analysis of genomic DNA from blasticidin- and puromycin-resistant polyclonal culture (*gsps*$^{-/-}$) and wildtype promastigotes. Primers used in PCR reactions are indicated in parenthesis. The same reactions were carried out in the absence of DNA to serve as negative control (C -).

Chapter 3

Discussion

1. General considerations

At present, chemotherapies used to treat visceral leishmaniasis are unsatisfactory. The quest for new molecules that efficiently induce parasite death is tightly correlated with the finding of new molecular targets that can be inhibited inside the host without undesired cross-reactivity. The thiol system of trypanosomatids is different from that of their mammalian hosts and has long been considered as a potential site for inhibition with specific drugs. This system largely depends on trypanothione, a unique dithiol that, directly or indirectly, supplies reducing equivalents to a myriad of enzymes, many of which have been proven essential for trypanosomatid survival. Therefore, targeting the enzyme responsible for the synthesis of trypanothione has been suggested as an adequate strategy to interfere with the thiol system of trypanosomatids, hence with parasite viability. Trypanothione synthetase is an exclusive, unique in sequence, low abundance protein that has been proven essential to *T. brucei*. However, this parasite lacks GSPS and, as such, the possibility of compensatory mechanisms involving mono(glutathionyl)spermidine remains unexploited in trypanosomatids. Validation of *TRYS* as a drug target in organisms harboring *GSPS*, such as *L. infantum*, has not been reported so far. The work carried out in the course of this Msc work focused on the dissection of the functional relevance of *TRYS* and *GSPS* in *L. infantum*. Our main findings and conclusions are discussed bellow.

2. *TRYS* is essential during the insect stage of *L. infantum*

Genetic validation of *TRYS* by homologous recombination proved that this gene is essential to *L. infantum* promastigotes, hence that mono(glutathionyl)spermidine alone cannot sustain all trypanothione-dependent functions. The first indication for *TRYS* essentiality came from our failure to generate homozygous knockout parasites. Indeed, the double targeted *HYG* and *PHLEO* transfectants, in addition to carrying both drug resistant markers, preserved the *TRYS ORF*. Although we did not explore this further, the presence of all three genes in these mutants may have occurred either by formation of extrachromosomal amplicons or by chromosome duplication, two strategies that are usually employed by *Leishmania* to avoid depletion of essential genes [*31*]. Elimination of both *TRYS* alleles was only possible upon complementation of heterozygous *trys*$^{+/-}$ mutants with an episomal copy of the gene. Definitive proof for *TRYS* essentiality was obtained from the observation that these pTEX-*NEO*-*TRYS*-complemented *trys*$^{-/-}$ promastigotes did not lose the plasmid even upon being cultured for 6 months in the absence of geneticin. In these transfectants the pressure to maintain the *pTEX-NEO* plasmid was provided by the requirement for the episomal *TRYS* gene during promastigote growth, thus proving that this is a crucial gene [*67*]. To assess *TRYS* essentiality in amastigotes

residing in mammalian hosts we employed an identical strategy. This experiment is still underway, but based on our finding that intramacrophagic *L. infantum* amastigotes are susceptible to FS-554, a potent inhibitor of TRYS *in vitro*, we expect this protein to be non-redundant in the amastigote stage as well.

3. *Li*TRYS can be targeted by drug-like compounds in the cell context

The chemical validation of *TRYS* was also pursued in this work using FS-554, a potent inhibitor of TRYS activity *in vitro* that belongs to the family of N^5-substituted paullones [*95*]. Assays performed on both promastigotes and intramacrophagic amastigotes revealed that TRYS expression levels consistently correlated with *L. infantum* resistance to FS-554. These observations strongly suggest that TRYS can be targeted in the cell context by drug-like compounds. From the rational drug design standpoint this is an important result as it evidences the possibility of designing drugs that can be internalized by parasites (as well as by macrophages) and bind TRYS. However, from our observations we cannot safely conclude that FS-554-induced parasite death is a consequence of TRYS inactivation. In fact, being aware that paullones interact promiscuously with most ATP-binding proteins [*96*], it may happen that FS-554 also inhibits other targets (*e.g.* GSPS, kinases) that are vital to the parasite. Following this rationale, overexpression of TRYS would prevent FS-554 from binding and inhibiting other potentially vital targets. One way to elucidate the inhibitory effect that FS-554 actually exerts over TRYS in the cell context involves the quantification of trypanothione (as well as other small thiols) in FS-554-treated *L. infantum* promastigotes.

4. What is the functional relevance of *GSPS*?

The presence, in some trypanosomatids, of a *GSPS* sequence is regarded as a trace of a common ancestor that is being lost throughout evolution. Supporting this idea is the fact that TRYS *per se* is capable of synthesizing trypanothione *in vitro*. Moreover, *L. major* carries a *GSPS* pseudogene that has been suggested to represent the evolutionary link between trypanosomatids that harbor both enzymes and those that possess only *TRYS* [*75*]. Despite these considerations, the relevance of *GSPS* in these organisms remains unaddressed, and this was the reason why we pursued the functional characterization of this protein in *L. infantum*. Upon the first attempt to generate *gsps*$^{-/-}$ knockouts, and while awaiting the outcome of this experiment, we can only speculate about the possible scenarios regarding *GSPS* essentiality. First, our results may indicate that *GSPS* is redundant throughout the *L. infantum* life cycle, and as such, confirm previous suggestions that this gene is to be lost throughout parasite evolution. Second, the enzyme may be found essential at least in one life cycle stage of the parasite. On the basis of

our current knowledge that TRYS is an essential enzyme or, in other words, that no other thiol can replace trypanothione, the functional relevance of GSPS cannot be attributed to its mono(glutathionyl)spermidine biosynthetic activity. Alternatively, an essential role for GSPS may be attributed to its putative ability to interact and form a functional complex with TRYS. In fact, as suggested by Oza *et al.* [*82*], in organisms expressing both proteins, GSPS, which is a homodimeric enzyme in *E. coli*, may associate with TRYS via several amino acid extensions that are absent in the prokaryotic enzyme. Formation of a functional TRYS-GSPS complex *in vivo* has so far been reported in *C. fasciculata* [*99*] and has not yet been addressed in other trypanosomatids harboring GSPS. In the future, if GSPS is found to be crucial for *L. infantum* survival, the heteromeric nature of TRYS and GSPS, as well as the influence that such putative complex has on the enzymatic activity of both proteins, will be investigated.

5. Final remarks

The work presented in this MSc thesis represents, to our knowledge, the first study of *TRYS* essentiality in a trypanosomatid harboring both *TRYS* and *GSPS* sequences. The results gathered here demonstrate that, at least in the promastigote stage of *L. infantum*, i) *TRYS* is crucial to ensure parasite survival and ii) as such, its functions cannot be replaced by *GSPS*. Work is on going to elucidate whether these observations also apply to the mammalian stage of *L. infantum*. In addition to these results, evidence is also provided that TRYS is a druggable target, *i.e.* it is prone to inactivation by drug-like compounds, a requisite for target validation. Finally, we also report on the production of *GSPS* knockouts, which will be used in future work to assess the functional relevance of this gene in *L. infantum*.

Chapter 4

Material & Methods

1. Parasite cultures

Leishmania infantum promastigotes (strain MHOM/MA/67/ITMAP-263) were cultured in RPMI 1640 Glutamax medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBSi), 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin and 20 mM Hepes sodium salt pH 7.4 (complete medium) at 25 ºC. Axenic amastigotes of the same strain were grown at 37 ºC in MAA medium supplemented with 20% FBSi, 2 mM glutamax (GibcoBRL®), 0.023 mM hemin as described previously [*67*]. When required, promastigote cultures were synchronized by 3–4 daily passages of 10^6 cells ml^{-1}. To obtain parasites at different stages of growth, cultures were seeded at 10^6 cells ml^{-1} and harvested daily from day 1 to day 6.

2. Primers

All primers used in this work to generate DNA constructs and/or to diagnose for their correct assembly and integration in the *L. infantum* genome are summarized in Table 4.1. Primers were purchased from Sigma-Alrdich® as non-modified, desalted oligonucleotids.

Table 4.1. List of oligonucleotides employed in this work. Clamp sequences are indicated in lower case and restriction sites underlined.

Primer name	Primer sequence	Function
P1	cccaagcttCTATATATGTGTCTCACTGC	Sense primer to amplify 5'NCS flanking *LiTRYS ORF*
P2	ggactagtGGCTTATGATTGCTGTGTG	Anti-sense primer to amplify 5'NCS flanking *LiTRYS ORF*
P3	cgcggatccCGTCCGGGCTGCAAGC	Sense primer to amplify 3'NCS flaking *LiTRYS ORF*
P4	cgcgagctcTGCTGCGCAATACACACAA	Anti-sense primer to amplify 3'NCS flaking *LiTRYS ORF*
P5	ccgcgcacatATGTCGTCTCTGCCG CG	Sense primer to amplify *LiTRYS ORF*
P6	caccgctcgagTTACTCGTCCTCGGCCAT	Anti-sense primer to amplify *LiTRYS ORF*
P7	CGCGGCAGAGACGACAT	Anti-sense primer at the beginning of *TRYS ORF*
P8	TCGTTGCTGGAGTACGATG	Sense primer to confirm correct integration *LiTRYS* replacement cassettes (gene upstream of *LiTRYS ORF*)
P9	CGCGGTGAGTTCAGGCTT	Anti-sense primer to confirm correct integration of *HYG* cassette (beginning of *HYG ORF*)
P10	CACTGGTCAACTTGGCCAT	Anti-sense primer to confirm correct integration of *PHLEO* cassette (beginning of *PHLEO ORF*)
P11	AAGCCTGAACTCACCGCG	Sense primer to amplify *HYG ORF*
P12	CTATTCCTTTGCCCTCGGA	Anti-sense primer to amplify *HYG ORF*
P13	ggactagtATGGCCAAGTTGACCAGTGC	Sense primer to amplify *PHLEO ORF*
P14	ggactagtTCAGTCCTGCTCCTCGGC	Anti-sense primer to amplify *PHLEO ORF*
P15	cccaagcttGCTACACATCAGCTGATGA	Sense primer to amplify 5'NCS flanking *LiGSPS ORF*
P16	cggactagtGAGCGTTTTTCGTAGCCG	Anti-sense primer to amplify 5'NCS flanking *LiGSPS ORF*
P17	cgcggatccCTCTCACCCTCGCTGTG	Sense primer to amplify 3'NCS flanking *LiGSPS ORF*
P18	gggagatctTGTGCGGTGCTGCAGGT	Anti-sense primer to amplify 3'NCS flanking *LiGSPS ORF*
P19	AAATCGTTCTGAGCGAGTAG	Sense primer to confirm correct integration of *LiGSPS* replacement cassettes (gene upstream of *LiGSPS*)
P20	CGCTGGCGACGCTGTAG	Anti-sense primer to confirm correct integration of *BSD* cassette
P21	TGGGCTTGTACTCGGTCAT	Anti-sense primer to confirm correct integration of *PAC* cassette
P22	GCGTGGCAGAGACAGCAT	Anti-sense primer at the beginning of *GSPS ORF*

P23	CTTGACGAGTTCTTCTGA	Sense primer to verify pTEX-*TRYS* integrity (end of *NEO ORF*)
P24	TTCAATGGCCGATCCCAT	Anti-sense primer to verify pTEX-*TRYS* integrity (beginning of *NEO ORF*)
P25	GTAAAA CGACGGCCAGT	Sense primer to verify pTEX-*TRYS* integrity (plasmid backbone)
P26	ACAGGAAACAGCTAT GA	Anti-sense primer to verify pTEX-*TRYS* integrity (plasmid backbone)
P27	ccggaattcATGGGATCGGCCATTGAA	Sense primer to amplify *NEO ORF*
P28	cgcggatccTCAGAAGAACTCGTCAAG	Anti-sense primer to amplify *NEO ORF*

3. Transformation of *Escherichia coli*

Escherichia coli DH5α competent cells, used in the generation of plasmid constructs, were obtained following the calcium chloride ($CaCl_2$) method [*100*]. Transformations were performed by incubating *E. coli* with 50-80 ng of DNA on ice for 30 min, followed by a heat shock at 42 ºC and incubation in SOC medium for 1 h at 37 ºC with shaking. Transformed cells were plated in LB-agar medium with 50 µg ml^{-1} ampicillin and incubated O/N at 37 ºC.

4. General cloning procedures

DNA fragments were PCR-amplified with the high fidelity *PWO* polymerase (Roche®) from the *L. infantum* genome and subsequently cloned into the appropriate vectors for propagation in *E. coli*. Assembling reactions were performed using T4 DNA ligase (Fermentas®) according to the manufacturer's instructions. Positive colonies were tested by PCR and the accuracy of the assembled constructs verified by DNA standard sequencing in Macrogen (Europe). Constructs were purified from *E. coli* using GenElute™ Plasmid Miniprep kit (Sigma®).

5. Generation of *TRYS* transfection constructs

Two DNA constructs carrying different drug resistance markers were generated to replace both *TRYS* alleles by homologous recombination. To that end, part of the 5' and 3' non-coding sequences flanking the *TRYS ORF* (5'NCS and 3'NCS, for short) were PCR-amplified from the *L. infantum* genome using the pairs of primers P1/P2 and P3/P4, respectively. Following digestion with the appropriate restriction enzymes, the 3'NCS was cloned into the *Bam*HI-*Sac*I sites of the pGL345-*TXN1*KØ and of the pGL726-*TXN1*KØ plasmids, previously used to target the *LiTXN*1 *ORF* [*67*], which carry the hygromycin phosphotransferase (*HYG*) gene and the phleomycin hydrolase (*PHLEO*) gene, respectively. The resulting constructs were subsequently digested with *Hind*III-*Spe*I restriction enzymes to accommodate the 5'NCS of *L. infantum TRYS*. Before transfection of *L. infantum* promastigotes, constructs were linearized with *Hind*III and *Sac*I and purified from agarose gels using the QIAquick® II Gel Extration Kit 250 (Qiagen).

The *TRYS* episome was generated by cloning the *TRYS ORF* into the trypanosomatid expression vector pTEX-*NEO* [*98*]. To that end, the *TRYS ORF*, previously cloned in our lab in frame with a 6-histidine tag in the *Nde*I/*Xho*I restriction sites of pET28c (Novagen®), was excised using the *Bgl*II/*Xho*I enzymes and cloned into the *Bam*HI/*Xho*I sites of pTEX-*NEO*. The resulting plasmid was subsequently digested with *Nde*I and *Eco*RI restriction enzymes followed by Klenow treatment and religation, in order to remove the 6-histidine coding sequence upstream the *TRYS ORF*.

6. Generation of *GSPS* transfection constructs

Two DNA constructs carrying different drug resistance markers were generated to replace both *GSPS* alleles by homologous recombination. Accordingly, part of the 5'NCS and 3'NCS flanking the *GSPS ORF* were PCR-amplified from the *L. infantum* genome using the pairs of primers P15/P16 and P17/P18, respectively. The 5'NCS was cloned into the *Hind*III-*Spe*I sites of the pGL434 vector, which carries the blasticidin S deaminase (*BSD*) gene, followed by insertion of the 3'NCS into the *Bam*HI-*Bgl*II sites. The second replacement construct was obtained by replacing the *BSD* gene in the previously assembled plasmid, by the puromycin N-acetyl-tranferase (*PAC*) gene, excised from pGL701 by *Spe*I-*Bam*HI restriction. Before transfection of *L. infantum* promastigotes, constructs were linearized with the restriction enzymes *Hind*III-*Bgl*II and purified from agarose gels using the QIAquick® II Gel Extration Kit 250 (Qiagen).

7. *Leishmania infantum* transfections

Transfections were performed in an Amaxa® Nucleofector™ (Lonza®), using the program U-033. Briefly, $5x10^7$ log-phase promastigotes, suspended in Human T cells nucleofactor buffer (Lonza®), were transfected with 2 to 10 µg of DNA and immediately transferred into flaks containing 10 ml of RPMI complete medium. These were then split into two 5 ml cultures and allowed to recover. Twenty four hours latter, selective drugs were added to 5 ml of the liquid cultures and the remaining 5 ml were pelleted and plated onto agar plates containing the same drug(s). Geneticin (G418; Sigma®) was used at 15 µg ml^{-1}, hygromycin (Invitrogen®) at 10 µg ml^{-1}, phleomycin (Sigma®) at 17.5 µg ml^{-1}, blasticidin S (Sigma®) at 30 µg ml^{-1} and puromycin (Sigma®) at 20 µg ml^{-1}. Upon 2 to 3 weeks of growth on agar, colonies were transferred into liquid medium and tested for the correct acquisition of the transfected DNA.

8. Cryopreservation of *L. infantum*

Promastigotes in logarithmic-phase of growth were suspended at $2x10^8$ cells ml^{-1} in RPMI medium supplemented with 20% FBSi, 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin and 15 % (v/v) dimethyl sulfoxide (DMSO). Cryotubes were placed in an isopropanol container previously cooled at 4ºC and incubated for 2 to 18 hours at -70 ºC before being stored in liquid nitrogen.

9. Preparation of genomic DNA from *L.infantum*

Two different protocols were used to extract genomic DNA from *L. infantum* parasites. For analysis of isolated clones Puregen Core Kit A (Qiagen) was used according to the manufacturer's instructions. For Southern blot, genomic DNA was isolated following a phenol/chloroform extraction protocol [*101*].

10. Southern blot

Southern blots were preformed according to standard protocols [*102*]. Total genomic DNA was digested with the *Sac*II restriction enzyme, resolved in 0.7% agarose gel and transferred onto Hybound-N+ nylon membrane (Amersham Bioscience®). Probes were labeled with [α-^{32}P] dCTP using RedPrime II Random Prime Labeling System (Amersham™ GE Healthcare) according to the manufacturer's instructions.

11. Western blot

L. infantum protein extracts were obtained by solubilizing 10^9 cells ml^{-1} in 1% (v/v) Nonidet P-40 (Sigma®) in PBS in the presence of a cocktail of protease inhibitors. Protein concentration in the extracts was determined by absorbance reading at 280 nm in Nanodrop spectrophotometer ND-1000. Extracts containing 1% β-mercaptoethanol were fractionated in a 10 % SDS-polyacrylamide gel electrophoresis and electroblotted onto Hybound-C+ nitro cellulose membranes (Amersham Bioscience®). The membranes were subsequently probed with anti-*Lm*TRYS primary antibody, kindly provided by Alan Fairlamb (University of Dundee, UK). Secondary antibody was ECL™ horseradish-conjugated anti-rat IgG (Amersham™ GE Healthcare). To control for sample loading, membranes were stained by incubation with Ponceau reagent for 15 minutes or with Sypro® Ruby Protein Blot Stain S-11791 (Molecular probes®) accordingly to manufacturer's instructions. Detection was performed using Super Signal® West Dura Extended Duration Substrate or Super Signal® West Pico Chemiluminescent Substrate detection reagents (Thermo Scientific).

12. Growth curves

For growth rate determination *L. infantum* promastigotes, previously synchronized, were seeded at 10^6 cells ml^{-1} and cell density monitored daily for 6 days with a hemocytometer.

13. N^5-substituded paullones

For the chemical validation of *TRYS* four compounds were selected from a collection of N^5-substituted paullones chemically synthetized and tested *in vitro* against purified recombinant *Li*TRYS by Molisa GmbH (Germany). All compounds were solubilized in 100% DMSO to a final concentration of 50 mM.

14. Isolation of murine peritoneal macrophages

Macrophages were obtained by peritoneal lavage of National Marine Research Institute (NMRI) mice from the animal house facility of the Institute for Molecular and Cell Biology (IBMC). To allow the formation of macrophages monolayers, $3x10^5$ cells per well were seeded in 24-well plates with round glass coverslips and allowed to adhere overnight in DMEM Glutamax™ culture medium supplemented with 20% FBSi, 50 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin (complete DMEM medium) at 37ºC in 5% CO_2 atmosphere. For some experiences, murine macrophages were seeded at $8x10^4$ cells per well in 96-well plates.

15. Leishmanicidal effect of N^5-substituded paullones on intracellular amastigotes

Monolayers of peritoneal murine macrophages were incubated with stationary-phase promastigotes (7-8 days culture) at a ratio of 10 parasites to 1 macrophage, for 3 hours at 37 ºC and 5% CO_2, in 24-well plates. Non-internalized parasites were removed by washing with pre-warmed DMEM medium and infected macrophages were cultured for 24 h in complete DMEM medium. To determine the effect of drugs in parasite survival, varying concentrations of FS-304, FS-554, FS-528 and FS-99a, were added to infected macrophages, keeping the DMSO concentration at 0,5% (v/v). Control cells were incubated without drugs with 0,5% (v/v) DMSO. Cells were grown for additional 48 h prior to being labeled for immunofluorescence analysis. To that end, monolayers of infected macrophages in round coverslips were fixed with methanol for 15 min at -20 ºC, permeabilized with 0.1% (v/v) Triton X-100 in PBS and blocked with 1% bovine serum albumin (BSA). Cells were incubated with a cocktail of primary anti-bodies (anti-*Li*cTXNPx1 + anti-*Li*mTXNPx [*62*]) for 1 h, washed with PBS, incubated for 1 h with the Alexa Fluor 488 anti-rabbit IgG (Molecular Probes) secondary antibody and for 15 min with a mixture of propidium iodide and 4',6-diamidino-2-phenylindole (DAPI). Samples were mounted in 70% (v/v) glycerol in PBS, examined with AxioImager Z1 fluorescence microscope and photographed with an Axiocam MR 3.0 using the Axiovision 4.6 software (Carl Zeiss). A

minimum of 4000 macrophages were counted per experimental condition using the CellNote software [*103*].

16. Detection of nitric oxide production in macrophages

Nitric oxide production was estimated indirectly by determination of nitrite (NO_2^-) concentration in macrophage cultures following the Griess assay [*104*]. Concentration of NO_2^- was determined in cultures supernatants by 1:1 addition of Griess reagent (1% sulfanilamide, 0.1% naphthalene-ethylenediamine dihydrochloride, 2.5% H_3PO_4) and measuring product formation (an Azo compound) spetrophotometrically at 550 nm. Increasing quantities of $NaNO_2$ were used as standards following the same treatment of samples.

17. Cell viability assays

Determination of cell susceptibility towards FS-554 was addressed using resazurin sodium salt (Sigma®). Fluorescence of the resorufin product was measured (excitation 560 nm; emission 590 nm) using a Spectra Max Gemini XS Microplate Spectrofluorometer (Molecular Devices®). Cell viability was determined considering the signal of cultures with 0 µM FS-554, 0,5 % (v/v) DMSO as 100% viability.

17.1. Promastigote viability

Synchronized promastigote cultures were seeded at 10^6 cells ml^{-1} in 24 well-plates in complete RPMI medium with varying concentrations of FS-554 and incubated for 48 h at 25 º C. Resazurin solution was added to a final concentration of 250 µM and fluorescence measured after 24 h, 48 h and 96 h. Data was analyzed with Graphpad prism 5 software and EC_{50} values determined with non-linear fit using a two-parameter equation:

$$\frac{}{}$$

17.2. Macrophages viability

Murine macrophages monolayers were seeded at $8x10^4$ cells per well in 96-well plates and allowed to adhere O/N in complete DMEM medium at 37 ºC and 5% CO_2. Varying concentrations of FS-554 were added and cells incubated for 48 h at 37 ºC and 5% CO_2. Resazurin solution was added to a final concentration of 125 µM and flourescense measured after 24h.

18. Geneticin-resistance assays

Promastigote cultures were seeded at 10^6 cells ml^{-1} in RPMI complete medium with 50 µg ml^{-1} G418 and without drug. Cells were counted 4-7 days after the addition of G418 and the data expressed as a percentage of parasite replication in control cultures without drug addition.

19. In vivo mice infections

BALB/c mice were infected by intra-peritoneal injection with 10^8 stationary-phase promastigotes in PBS. Animals were sacrificed at determined time points after infection and their livers and spleens homogenized in complete RPMI medium, incubated at 25 ºC and differentiated promastigotes cultured for further analysis.

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